Volume 24, No. 1

January-February 1959

BIOCHEMISTRY

БИОХИМИЯ

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THE LIGHT REACTIVATION OF PLANT TISSUE CYTOCHROME OXIDASE ACTIVITY IN THE PRESENCE AND ABSENCE OF CHLOROPHYLL

N.M. Sisakyan, A.A. Krasnovskii, E.S. Mikhailova and G.P. Brin

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In the chloroplasts of plant leaves there are different enzyme systems which can take part in the process of photosynthesis. For a study of the possible paths by which the enzyme systems can take part in the photosynthetic chain reaction, we can use the method of selective reversible inhibition of the enzyme system with a study of the action on respiration and photosynthesis. In homogenates of green leaves it is possible to study these actions on that part of the reaction of photosynthesis which accompanies the photochemical evolution of oxygen, the Hill reaction.

In our previous study we investigated the effect of concurrent inhibition of the dehydrogenases by nicotinic acid amide and found a correlation between suppression of the activity of these enzymes and the photochemical processes [1].

Among the enzyme systems which may be connected with the processes of photosynthesis [2] it is interesting to study the cytochrome oxidases which are especially widely distributed in the tissues of higher plants [3].

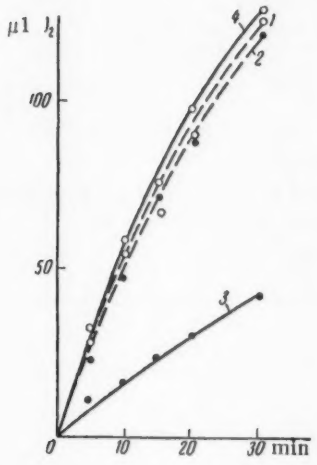

Fig. 1. Cytochrome oxidase activity of bean leaf suspensions: 1 and 2) in an atmosphere of $N_2 + O_2$; 3 and 4) in an atmosphere of $CO + O_2$; ●) in the dark; ○) in the light.

In this work we have studied the effect of carbon monoxide on cytochrome oxidase and the photochemical activity of plant leaf homogenates. Carbon monoxide reacts with the iron porphyrin of cytochrome oxidase to form a complex which is decomposed by visible light [4].

The possibility of reactivation of the processes suppressed by carbon monoxide was studied under the action of "white" light which is absorbed by cytochrome oxidase and chlorophyll, and red light, which is absorbed only by chlorophyll.

For the determination of cytochrome oxidase and photochemical activity we used the manometric method. The experiments were carried out at 24-25°. The cytochrome oxidase activity was estimated by the amount of oxygen absorbed in oxidation by reduced hydroquinone of cytochrome c (10 mg of hydroquinone per 1 ml of $1.80 \cdot 10^{-4}$ M cytochrome c). As an inhibitor of the copper-containing oxidase we used 0.014 M sodium diethyldithiocarbamate. Photochemical activity was measured by the reduction of phenol indophenol [5].

The leaf sample was ground in the cold with 1/15 M phosphate buffer, pH 7.2, and centrifuged 1-3 min at 2000-2500 rpm. The resulting suspension was used for the experiment.

For the determination of cytochrome oxidase activity, in the main part of the manometric vessel was placed 2 ml of the suspension, in the central flask 0.3 ml of a 20% KOH solution, and in the side arm 0.5 ml

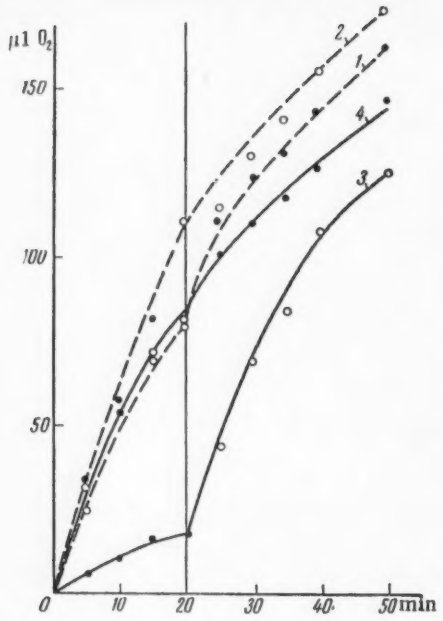

Fig. 2. Cytochrome oxidase activity of goosefoot leaf suspensions: 1 and 2) in an atmosphere of $N_2 + O_2$; 3 and 4) in an atmosphere of $CO + O_2$; ●) in the dark; ○) in the light.

of reduced cytochrome c and 0.5 ml of sodium diethyldithiocarbamate.

For the determination of photochemical activity, we placed 3 ml of the suspension in the Warburg vessel and 1 ml of phenol indophenol saturated at room temperature (0.01 M) in the side arm. The experiments were carried out in an atmosphere of 90% CO and 10% O_2. As a control we used a vessel which contained 90% N_2 and 10% O_2. The gas mixture was passed through the vessel for 20 minutes, 1 liter for each vessel, which corresponded to 40 times the capacity of the vessel. While the gas was passing, the vessel was in a thermostat.

In the determination of cytochrome oxidase activity, one pair of vessels (one with an atmosphere of $CO + O_2$, the other with $N_2 + O_2$) was kept in the light while the gas mixtures were passed through, another pair was kept in the dark. Illumination was furnished by an incandescent lamp (200 w at a distance of 6 cm from the lamp filament to the bottom of the vessel, about $2 \cdot 10^{-4}$ erg/cm^2 · sec) without a light filter or through a red light filter RG-2. In determining the photochemical activity, the gas mixture was passed through the vessel in the dark.

Cytochrome oxidase activity was determined over 30-40 min. The photochemical activity took 21 min in red light.

We studied suspensions of the leaves of beans, beets, goosefoot, clover, wheat, potato, and nasturtium. In all cases we found suppression of cytochrome oxidase activity with carbon monoxide in the dark, and absence of suppression in the light. For example, in Fig. 1 we give the results obtained with suspensions of bean leaves.

Figure 1 shows that light has no effect on the activity of cytochrome oxidase suspensions in an atmosphere of $N_2 + O_2$. In a carbon monoxide atmosphere in the dark there is considerable decrease in activity of the enzyme. When it is illuminated with white light, the cytochrome oxidase does not show inhibition of activity with carbon monoxide; the activity is close to that in the $N_2 + O_2$ atmosphere.

Photochemical activity is not suppressed by the action of carbon monoxide. The reduction of phenol indophenol, which we observed by the evolution of oxygen, was the same in the $N_2 + O_2$ and $CO + O_2$ atmospheres.

In another series of experiments after 20 min measurement of the cytochrome oxidase activity, the manometers were exchanged: that which had stood in the dark was transferred to the light and that which was in the light was placed in the dark; then measurement of the activity was continued for another 30 min.

In this case we found not only an absence of inhibition in the light, but also a reactivation of cytochrome oxidase activity which had been suppressed by carbon monoxide (Fig. 2).

Figure 2 shows that after the suppression of cytochrome oxidase activity of goosefoot leaf suspensions in the dark (curve 3) it is reactivated by white light.

Since the determination of photochemical activity was carried out in red light, we studied the effect of this light on the suppression of carbon monoxide cytochrome oxidase activity in green leaf suspensions (Table 1). We showed that in red light there is an increase in cytochrome oxidase activity suppressed by carbon monoxide, though to a lesser extent than in white light.

The studies of the action of red and white light were carried out separately, and in each case dark and light controls were run in an atmosphere of $N_2 + O_2$; it should be mentioned that the control experiments in the nitrogen atmosphere in the light differed from the dark experiments by not more than 10%, which did not exceed the experimental error.

TABLE 1

Action of Carbon Monoxide, Red and White Light on Cytochrome Oxidase Activity. Activity in % of Control ($N_2 + O_2$) Taken as 100

Plant	$CO + O_2$, dark	Experimental conditions		
		$CO + O_2$, red light	$CO + O_2$, dark	$CO + O_2$, white light
Goosefoot	22	62	22	71
Beet	31	49	50	72
Bean	22	72	33	91
Potato	23	35	30	58
Nasturtium	33	100	21	92
Clover	–	–	29	68

For 100% activity of the cytochrome oxidase we used the average value of the control experiments (dark and light) after 20 min, expressed as μliters of oxygen. Although cytochrome oxidase did not absorb light in the region passed by the red filter* we found a reactivation of cytochrome oxidase activity in the green suspensions which had been suppressed by carbon monoxide, a reactivation by the action of red light which was close to the degree of reactivation by white light.

In order to explain the reason for this effect, we ran a comparative study on plants in different physiological states, with or without chlorophyll. We used sprouts and green leaves of potatoes; milky seeds, etiolated and green sprouts of wheat. The apparatus used in this series of experiments did not differ essentially from that described before, except that in the exchange of the vessels to the light from the dark (and vice versa) the stopcocks were closed and later readings were again taken from the null position.

The results are given in Figs. 3 and 4, and in Table 2.

TABLE 2

Action of Carbon Monoxide, Red and White Light on the Cytochrome Oxidase Activity of Plants Which do not Contain Chlorophyll Activity in % of Control ($N_2 + O_2$) Taken as 100

Objects of study	$CO + O_2$, dark	Experimental conditions		
		$CO + O_2$, red light	$CO + O_2$, dark	$CO + O_2$, white light
Potato sprouts	24	24	51	100
Milky wheat seeds	37	50	36	79
Etiolated wheat sprouts	20	27	20	49

As a result of these experiments we showed that white light reactivates the cytochrome oxidase whose activity is suppressed by carbon monoxide in green suspensions and in suspensions which do not contain chlorophyll (curve 4, Figs. 3 and 4,A). Red light has a reactivating action only on the cytochrome oxidase activity of suspensions from etiolated sprouts and wheat seeds (Table 2) can evidently be explained by the presence in them of protochlorophyll, which during the experiments is transformed into chlorophyll by scattered light.

Thus, these experiments show that white light reactivates cytochrome oxidase whose activity has been suppressed by carbon monoxide in green suspensions and in suspensions which do not contain chlorophyll. Red light reactivates cytochrome oxidase whose activity has been suppressed by carbon monoxide only in suspensions which contain chlorophyll; in suspensions which do not contain chlorophyll, reactivation by red light does not occur.

We cannot judge the action of CO on photochemical activity in green leaf suspensions, since the presence

*The oxidized form of the purified preparation of cytochrome oxidase from pig heart muscle had a maximum absorption at 595 mμ (principal) and at 420 mμ (weak), and the reduced form at 605 and 445 mμ [6].

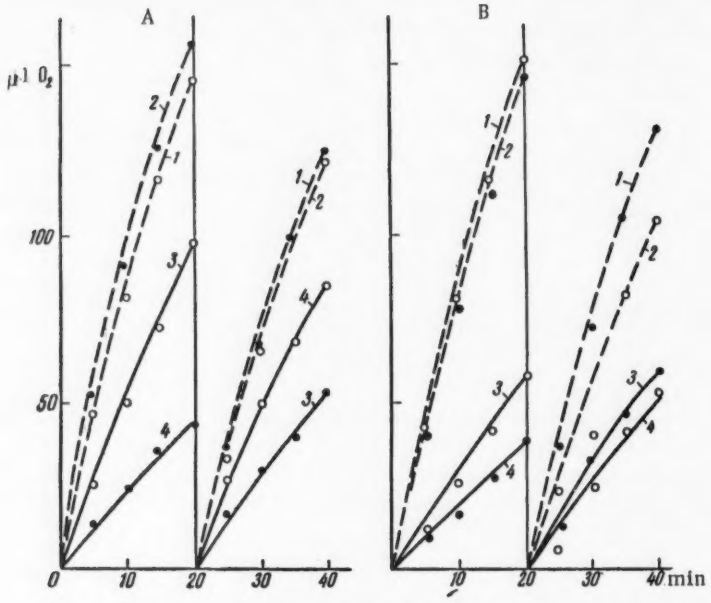

Fig. 3. Action of white (A) and red (B) light on the cytochrome oxidase activity of potato-leaf suspensions: 1 and 2) in $N_2 + O_2$ atmosphere; 3 and 4) in $CO + O_2$ atmosphere; ○) in light; ●) in dark.

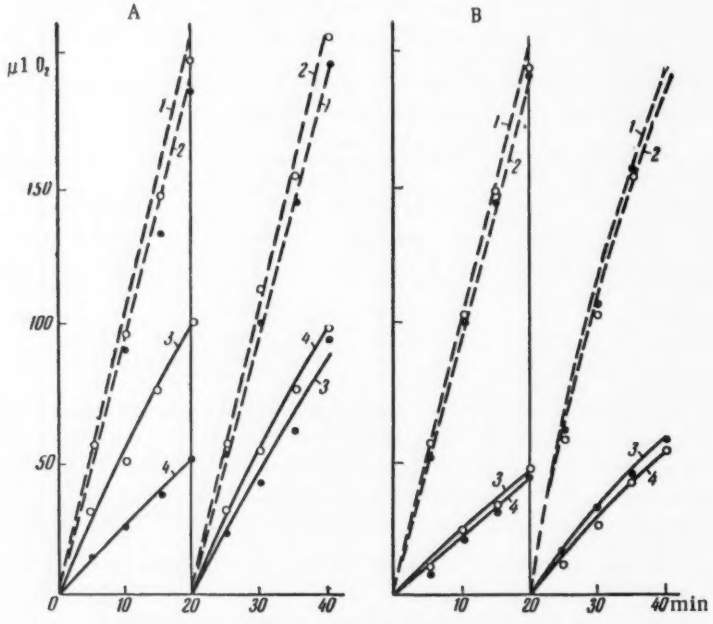

Fig. 4. Action of white (A) and red (B) light on cytochrome oxidase activity of potato-sprout suspension: 1 and 2) in $N_2 + O_2$ atmosphere; 3 and 4) in $CO + O_2$ atmosphere; ○) in light; ●) in dark.

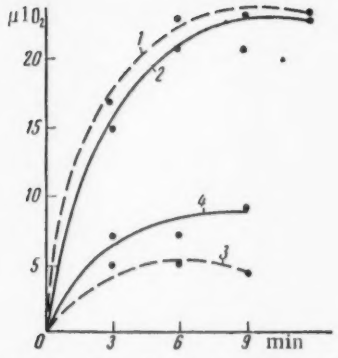

Fig. 5. Photochemical activity of suspensions of leaves (1 and 2) and sprouts (3 and 4) of potatoes: dashed line – in a $N_2 + O_2$ atmosphere; solid line – in a $CO + O_2$ atmosphere.

of chlorophyll permits reactivation of cytochrome oxidase whose activity has been suppressed by carbon monoxide in red light.

Figure 5 shows that the photochemical activity in a $CO + O_2$ atmosphere does not differ from that in a $N_2 + O_2$ atmosphere.

Thus, our experiments show that the presence of chlorophyll determines the reactivation by light of cytochrome oxidase whose activity has been suppressed by carbon monoxide. It is possible that this effect is similar in mechanism to the photosensitization of the oxidative-reductive transformation of cytochrome c under the action of light absorbed by chlorophyll [7].

We can make the following assumptions about the mechanism of the effect which we have studied:

1) the energy of a light quantum absorbed by chlorophyll is used for dissociation of the complex of cytochrome c oxidase with carbon monoxide;

2) increased absorption of oxygen when the homogenates are illuminated by red light is determined not only by reactivation of cytochrome oxidase, but also by photosensitization by chlorphylll of the oxidation of reduced cytochrome.

The experiments described in this paper do not now permit a definite conclusion as to the mechanism of the observed effects; studies in this direction are being continued.

SUMMARY

To explain the connection between photochemical and cytochrome oxidase activity of green leaf homogenates we have used the method of inhibiting cytochrome oxidase activity by carbon monoxide and its reactivation by light. White light reactivates the cytochrome oxidase of leaf suspensions with and without chlorophyll, after its activity has been suppressed by carbon monoxide. Red light reactivates such cytochrome oxidase only in the green tissues of plants. Thus, our experiments show that the presence of chlorophyll in the tissues determines the reactivation of cytochrome oxidase, whose activity has been suppressed by carbon monoxide when red light is used.

LITERATURE CITED

[1] N.M. Sisakyan, A.A. Krasnovskii, E.S. Mikhailova and G.P. Brin, Biokhimiya 18, 725 (1953).

[2] C.S. Gocum, Amer. J. Bot. 33, 10 (1946).

[3] E.F. Hartree, Advances Enzymol. 18, 2 (1957).

[4] O. Warburg, Biochem. Z. 177, 471 (1926).

[5] A.S. Holt and C.S. French, Arch. Biochem. 19, 429, 148.

[6] L. Smith and E. Stetz, J. Biol. Chem. 209, 819 (1954).

[7] A.A. Krasnovskii, Doklady Akad. Nauk SSSR 103, 283 (1955); A.A. Krasnovskii and K.K. Voinovskaya, Biofizika 1, 120 (1956).

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SCUMPIA TANNINS

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The chemical nature of scumpia tannins has not been specifically studied, if we do not consider several works which classify them and consider possible uses [1, 2]. The sumacs, related to scumpia, have been studied in more detail, but there is still not complete understanding of even sumac tannins.

Most of the tannin complex of these plants consists of a mixture of gallic acid derivatives of sugars (gallotannins), but the composition of the mixture and the structure of the separate components has not yet been worked out. In many cases the literature descriptions are vague and contradictory.

Münz [3] studied the tannins removed by ethyl acetate from a neutralized aqueous extract of sumac leaves. When he split the product with a tannase, he found 88% gallic acid and 8.2% glucose. Alkaline hydrolysis of the methylated tannins gave trimethyl gallic acid. On the basis of these results and elementary analyses, the author drew the hasty conclusion that sumac tannin is pentagalloyl-β-glucose.

A more detailed study of sumac by Kirby and Catravas [4] gave a tannin extracted by acetone from leaves which had first been freed from chlorophyll and polyphenols by extraction with chloroform and ether. Later precipitation with aluminum hydroxide gave five tannin fractions which differed in values of specific rotation. Methoxy, acetyl and bromo derivatives, elementary analysis, and the results of hydrolysis of the first and fifth fractions showed a close agreement with m-digalloyl-tri(galloyl)-glucose and di(m-digalloyl)galloyl-glucose. The authors considered sumac tannins as a mixture especially of these two tannins. In distinction from Münz, they considered that the alcoholic hydroxyl of glucose was esterified not only with gallic, but partly also with digallic acids.

Comparatively recently Stiefenhofer et al. [5] have studied sumac tannins. In this work they have used, in addition to the classical methods, electrophoresis and chromatography. The tannins which were extracted by ethyl acetate from a water solution at pH 3.5 and 7 were separated into two parts, acid and neutral. The authors considered, without sufficient basis, that these two fractions were identical and limited their characterization only to the neutral part, which they methylated and then hydrolyzed. In the hydrolyzed product from the methylated tannins they found trimethyl and dimethylgallic acids in the ratio 2:1. In the tannin carbohydrates after purification by electrophoresis and further hydrolysis by sulfuric acid they detected, by paper chromatography, glucose, rhamnose and arabinose in the ratio 2:1:1, which, in their opinion, were combined in the original molecule of tannin as a tetrasaccharide. They considered sumac tannin as a tetrasaccharide in which twelve free hydroxyl groups were esterified by gallic and digallic acids.

Here, in our view, is a methodological error: it is not necessary to ascribe all the complicated tannins of the complex to one substance. On the other hand, the carbohydrate components require further study since it is possible that the other sugars are not part of the tannin, but are only mixtures which cannot be separated from the methylated product.

We set ourselves the problem of making a more detailed study of the five phenolic substances which had earlier been detected in scumpia leaves [6].

EXPERIMENTAL

Isolation of the tannins. The tannins were extracted from air-dried, ground scumpia leaves (collected in June in the region of Kishinev) by standing three times in water at room temperature. The filtered-water extract was extracted with a mixture of butyl acetate and n-butanol in the ratio 4:1 by volume (to 1 liter of water solution was added 80 g NaCl for salting out and 300 ml of solvent). The resulting extract was treated with an equal volume of water and the butyl acetate was distilled off under vacuum as an azeotropic mixture at 50-60°. The water solution of tannins was evaporated under vacuum to the consistency of a thick syrup and drying was completed over P_2O_5.

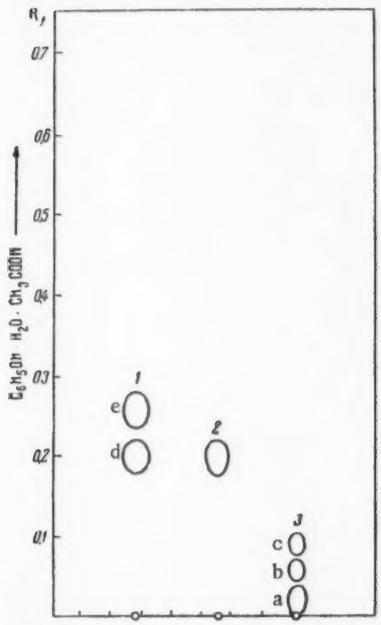

Fig. 1. Chromatogram of the fractions of tannin isolated from scumpia leaves: 1) ether-soluble fraction; 2) gallic acid; 3) ether-insoluble fraction.

Separation of polyphenols from tannins. The resulting tannin preparation was separated into two fractions: soluble and insoluble in ether. 50 g of tannin was dissolved in 1 liter of water and the resulting solution was extracted until an ether extract ceased to give a color with a solution of $FeCl_3$. The ether extract was dried with anhydrous Na_2SO_4 and the solvent distilled off to dryness on a water bath in a stream of CO_2. The residue was a powder of a dirty yellow color (the polyphenol fraction). The yield was 4.6 g or 9.2% of the original preparation. To the remaining water solution we added butyl acetate and the solvent was distilled off in a vacuum at 40-50° in a stream of CO_2. The residual water was removed by drying in a vacuum over P_2O_5. The resulting preparation was a yellow-brown, amorphous powder (the tannin fraction).

The polyphenol and tannin fractions were separated by chromatography on Whatman No. 1 paper in a solvent of phenol saturated with water with addition of 5% acetic acid. The developer was a 1% $FeCl_3$ solution.

In Fig. 1 we show that in the tannin fraction, insoluble in ether there are three substances present (a, b and c) and in the polyphenol fraction, two (d and e).

Separation and identification of the polyphenols. Separation of the polyphenols was carried out on a column of cellulose powder. The cellulose powder was prepared as follows: sheets of chromatographic paper were kept for 24 hours in 2 N HCl. Then they were washed repeatedly with water to full removal of Cl ion, dried, and ground into a powder. Sifting through a sieve (diameter of openings 0.25 mm) removed the most finely dispersed particles and the column was filled. Thirty g of cellulose powder was packed into the column which was half full of solvent and when the stopcock was opened at a pressure of 8-10 cm Hg, the paper was packed into the column for 25-30 min. Then the pressure in the column was reduced, and for 10 min more solvent was passed without pressure. A layer of solvent 1-2 mm thick was poured above the surface of the adsorbent, the solution under study was poured into the column, and the column was washed with the solvent. Control of the eluate was carried out with a 1% $FeCl_3$ solution and a 2% KCN solution. As a solvent we used acetic acid, prepared by mixing seven parts by volume of glacial acetic acid with three parts of distilled water.

As a result of the chromatography we obtained three fractions; from two of them (I and III) we isolated individual crystalline substances with melting points 357 and 235°, which were identified as myricetin and gallic acid.

For the separation 1 g of the polyphenol fraction was dissolved in 15 ml of acetic acid, poured into the column, and washed with the solvent.

We collected three fractions: I – 70 ml; II – 40 ml; III – 100 ml.

From fraction I after evaporation in a vacuum dark yellow crystals separated and after recrystallization from 70% alcohol and drying in a vacuum at 100° over P_2O_5, they melted at 355-357° with decomposition.

With a chloroform solution of $SbCl_3$ the substance gave a yellow-orange color. In 10% alkali the color changed from yellow to green, blue, and blue violet.

Substance – 15.00 mg; CO_2 – 31.04 mg; H_2O – 4.14 mg. Found C – 56.46%; H – 3.06%. Calculated for myricetin: C – 56.6%; H – 3.18%.

From fraction III after evaporation crystals precipitated which after recrystallization from water melted at 235°. A mixed melting point with gallic acid showed no depression.

TABLE 1

Calibration of the Electrophotocolorimeter Readings for Content of OH Groups

Scale reading	OH, μg	Scale reading	OH, μg	Scale reading	OH, μg	Scale reading	OH, μg	Scale reading	OH, μg
25	253	35	214	45	174	55	136	65	97
26	250	36	210	46	170	56	132	66	93
27	246	37	206	47	167	57	128	67	89
28	242	38	202	48	163	58	124	68	85
29	238	39	198	49	160	59	120	69	81
30	234	40	194	50	156	60	116	70	77
31	230	41	190	51	152	61	113	71	74
32	226	42	186	52	148	62	109	72	70
33	222	43	182	53	144	63	105	73	66
34	218	44	178	54	140	64	101	74	62

TABLE 2

Content of OH Groups in the Separate Tannin Components

Component	Concentration of solution, mg liter	Taken for determination, ml	Substance in given volume, μg	Scale reading	OH, μg	OH, %	Average
		5	800	40.5	192	24	
a	200	6	960	32	226	23.6	23.3
		7	1120	26	249	22.2	
		4	332	43.5	180	21.8	
b	208	5	1040	34	218	20.9	20.9
		6	1248	26	249	20	
		5	940	49	160	17	
c	188	6	1128	41.5	188	16.7	16.9
		7	1316	36	210	16	

Separation of the tannin fraction. For separation of the tannin fraction we used chromatography on many sheets of paper followed by cutting out strips and washing out the separate components with methanol. On sheets of chromatographic paper No. 4, first washed with N HCl and water to full removal of Cl and Fe, and with dimentions 45 x 45 cm, we placed a strip 0.5 cm wide of an alcoholic solution of the tannins at a distance of 5 cm from the bottom edge. The paper was rolled into a cylinder and placed in a vessel with the solvent (phenol saturated with water with 5% glacial acetic acid). Chromatography was carried out in an ascending stream of solvent.

After removal of the solvent by air drying, thin strips were cut from the left and right edges of the chromatogram and these were developed with $FeCl_3$. Corresponding to the positions of the separated substances each chromatogram was divided into three parts which contained the individual components a, b and c (Fig. 1). Extraction was carried out with methanol which was then distilled off in a vacuum in a stream of nitrogen and at

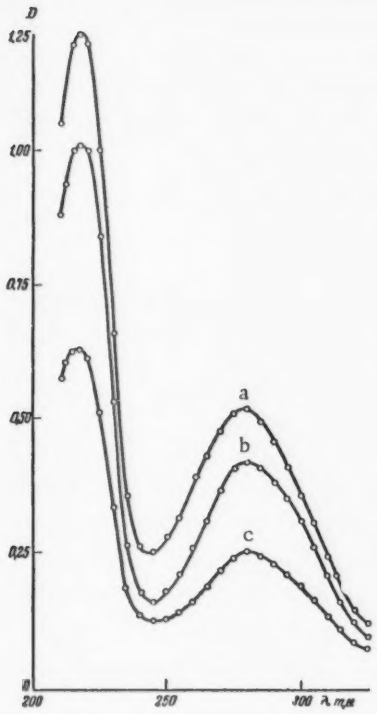

Fig. 2. Ultraviolet absorption spectra of components a, b and c.

the end of the distillation a small amount of water was added to complete the removal of the alcohol. After distillation of the solvent, the substances were dried in a vacuum over P_2O_5 at 100°.

In all, we obtained 0.17 g of substance a, 0.1 g of b, and 0.13 g of c.

Determination of the percent content of OH groups. In the resulting substances, which were amorphous powders, we determined the quantity of glucose hydroxyls colorimetrically according to Zaprometov [7].

The determination was carried out in an electrophotocolorimeter FÉK M-1. In a 50 ml measuring cylinder we placed several milliliters of the substance being studied, 5 ml of 0.1 M phosphate buffer with pH 6.24, and 2 ml of ferrous tartrate solution (0.18 g $FeSO_4$ and 0.67 g of Rochelle salt in 100 ml of H_2O). The volume was brought to 50 ml with distilled water, carefully mixed, and after 5 min determined in the colorimeter with a red filter, in a cuvette 5 cm thick. In the control cuvette we placed the same solution but without the reagent. Recording was carried out on a dark scale drum. On the basis of a series of measurements for solutions of different concentrations of well-purified gallic acid or pyrogallol we set up a calibrated table (Table 1) in which the scale readings were expressed directly in μg of hydroxyl groups.

The calibration table was set up for the interval of scale readings from 25 to 75 divisions, where the results of determination of hydroxyl groups were more accurate and the error of the determinations did not exceed 2%.

We dissolved 4-6 mg of each of the components a, b and c in 5 ml of methanol and diluted the solution with distilled water to 25 ml. From the solution with known concentration in the cylinder we took a definite volume, added the buffer and the reagent, and ran in the colorimeter.

The results of the determination of the hydroxyls in the tannin components of the complex showed that in substances a, b and c there were 23.3, 20.9, and 16.9% OH groups (Table 2).

These figures are very close to the calculated values of hydroxyl groups for di(galloyl)digalloyl-glucose, galloyldigalloyl-glucose, and digalloyl-glucose, which are 23.7, 21.4, and 17.5%, respectively.

Ultraviolet absorption spectra of the components. The absorption spectra were measured on a Beckman spectrophotometer SF-4 at a 1-cm thickness of absorbing layer. We studied an alcoholic solution of components a, b and c in 0.002% concentration. The absorption curves are given in Fig. 2.

In the ultraviolet spectra for all the components we found two entirely similar maxima at wavelength 217 and 280 mμ, which indicates a similar structure for all the components. The absorption maximum at 217 mμ is characteristic for the phenolic hydroxyls of gallic acid. The second maximum is evidently connected with the presence of a benzene ring. Benzene has an absorption maximum at 260 mμ. Apparently, under the influence of the substituent groups this maximum shifts toward the longer wavelengths, as Gillem and Stern [8] have shown. In such compounds as benzaldehyde, p-hydroxybenzaldehyde, vanilin, and tannins [9, 10], the carbonyl groups of which are bound to the ring, the absorption maxima are close to 280 mμ. In our case, this maximum depends on the galloyl residues bound to glucose. In such compounds as pyrocatechin and pyrogallol, which do not contain carbonyl groups, we find "benzene absorption" with maximum at wavelength 260 mμ [11].

As Fig. 2 shows, the density of absorption both at 217 mμ and 280 mμ rises regularly from component c to component a at about the same value, which is probably connected with the increased number of molecules in the gallic-acid residues. To test the correctness of this conclusion, we must study the absorption of components with the same molar concentration. Therefore, each of the components was methylated with diazomethane, and for the methyl derivatives we determined the molecular weight by the Rast method.

The observed values for component a (955), b (728), and c (503) were close to those calculated for the methyl derivatives of tetra-, tri-, and digalloylglucose, which were 942, 748, and 554, respectively.

For the methyl derivatives of substances a, b and c at concentrations of $4 \cdot 10^{-5}$ mole/liter and thickness of layer of 1 cm we determined the ultraviolet absorption spectra. For all three components we found only one maximum at wavelength 270 mμ. The optical density (D) at the maximum point for component a was 1.15, for component b, 0.898, and for component c, 0.607. Thus, the intensity of absorption rose regularly from component c to component a at the same value.

The coefficient of molar absorption (ε) calculated for one galloyl residue by the formula

$$\epsilon = D/(c \cdot n),$$

where c is the molar concentration and n the number of galloyl groups, starting from the observed molecular weights, is: $\epsilon(a) = 7.2 \cdot 10^3$; $\epsilon(b) = 7.43 \cdot 10^3$; and $\epsilon(c) = 7.59 \cdot 10^3$, that is, practically the same. This shows conclusively that the tannin components are tetra-, tri-, and digalloylglucoses.

SUMMARY

By chromatography on paper and on a column filled with cellulose powder the tannins of scumpia were separated into five individual substances, of which three were part of the tannin complex. In the polyphenol fraction of the tannins two substances were separated and identified as myricetin and gallic acid.

The tannin consists of three components: a, b and c. According to the results of content of OH groups, molecular weights and ultraviolet absorption spectra, these substances are galloyl derivatives of glucose with a content of 2, 3 and 4 gallic-acid residues.

LITERATURE CITED

[1] P. Yakimov and K. Ul'man, Trudy po Prikl. Botan. Genet. i Selektsii 1, 55 (1933).

[2] Ya. Mazover, Med. Prom.-st. SSSR 1, 36 (1947).

[3] W. Münz, Collegium. 499 (1929).

[4] G. Catravas and K. Kirby, J. Soc. Leather Trades' Chemists 32, 155 (1948).

[5] G. Stiefenhofer, H. Endress and W. Grassman, Chem. Ber. 89, 454 (1956).

[6] G. Lazur'evskii, D. Novokhatka and R. Roitburd, Uchen. Zap. Kishinevskogo un-ta 23, 85 (1956).

[7] A. Kursanov and M. Zaprometov, Biokhimiya 14, 466 (1949).

[8] A. Gillem and E. Stern, Electron Absorption Spectra of Organic Compounds [Russian translation] (Izd. Inostran. Lit., Moscow, 1957) p. 161.

[9] I. Egorov and N. Borisova, Sb. Biokhimiya Vinodeliya No. 5, 27 (1957).*

[10] A. Mikhailov, Chemistry of Tannins and the Tanning Process [in Russian] (Gizlegprom., Moscow, 1953).

[11] A. Onikienko, Collection: Application of Spectroscopic Methods in Industry and Agriculture of Consumer Goods [in Russian] (Izd. LGU, 1957) p. 180.

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THE AMPEROMETRIC DETERMINATION OF SULFHYDRYL GROUPS IN THE PROTEOLYSIS OF GRAIN

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The proteolytic activity of grain determines to a considerable extent its technological properties. In bread baking an understanding of the proteolytic activity permits control of the process of preparing the dough from strong or weak wheats, and in brewing, the proteolytic activity of barley and malt determines the amount of protein and products of its hydrolysis which are found in the extracts and in the beer.

A number of investigators [1-3] have shown that the proteolysis of sulfur-containing proteins, including those of grain, is accompanied by increase in the concentration of active sulfhydryl groups. It has also been established that compounds which contain sulfhydryl groups, especially substances of the type of glutathione, play a primary role in the metabolism of plant cells and are the natural activators of proteolysis. Therefore, the determination of the concentration of sulfhydryl groups in the proteolysis of grain is of great interest.

The quantitative determination of sulfhydryl groups in grain and flour is made difficult by the fact that their concentration is very low and therefore an extremely sensitive method is required. The method of iodometric titration is most often used [4, 5, 6]. Recently, in biochemical investigations, polarographic and amperometric methods have been used for the quantitative determination of SH groups [7].

Thus, a polarographic method for determination of the content of SH groups in wheat proteins has been described by De Large and Hintzer [8]. In testing the method on a protein fraction prepared from flour obtained from thirteen types of wheat, it was shown that the content of SH groups calculated on cystine varied for gluten in the limits 0.28-0.61% and for soluble proteins, 0.46-2.5%.

Kolthoff, Stricks and Morren [9] described an amperometric method for determining SH groups in different protein materials, including wheat flour, by titration with mercuric chloride. Matsumoto [10] determined SH groups in gluten by amperometric titration with silver nitrate.

Benesch, Lardy and Benesch [11] studied the amperometric titration of SH groups and showed that at high pH values of an ammonia buffer which was usually used for titration with silver nitrate, it was possible to denature the protein and oxidize part of the SH groups, and also partly to combine them with ammonia ions. Therefore, instead of an ammonia buffer, they used tris-(hydroxymethyl)aminomethane which forms a complex compound with silver ions.

Kong, Mecham and Pence [12] made a careful comparison of the methods of determining sulfhydryl groups in wheat flour: titration by o-iodosobenzoate, amperometric titration by mercuric chloride and silver nitrate, including the improved method of Benesch et al. [11]. They showed that the iodosobenzoate method gives high results, since with it the oxidation of the SH group goes beyond the disulfides, and also the lipides of the flour are oxidized. Amperometric titration by $HgCl_2$ or $AgNO_3$ is more preferable, both in sensitivity and accuracy of measurement, and the results of the measurements in these cases are more reproducible and objective in determining the end point of the titration. They used the amperometric method for the determination of the content of sulfhydryl groups in four types of wheat flour (in phosphate extracts) and found 0.18-0.30 μg-equiv SH groups per g of flour.

Smith, van Buren and Andrews [13], studying the action of oxygen and fat on the physical and chemical properties of wheat dough, determined the content of SH groups by the iodosobenzoate method in dough prepared from different types of wheat flour in atmospheres of oxygen and nitrogen, and also in dough from defatted flour. They showed that dough from poorer types of flour contained more sulfhydryl groups and that when the dough was kneaded in a nitrogen atmosphere the quantity of SH groups decreased, in less degree in dough from defatted flour of the better sort. These experiments confirmed the fact that the fat of flour is involved in oxidative reactions. Evidently, the choice of the iodosobenzoate method for determining the SH groups in this case was not fortunate, as the work of Kong et al. showed [12].

EXPERIMENTAL

After a consideration of all the above literature data, we chose for the determination of SH groups the method of amperometric titration with a rotating platinum electrode, as suggested by Kolthoff and Harris [14].

As a result of comparative experiments with $AgNO_3$ and $HgCl_2$ we showed that in titrations with a rotating platinum electrode, the silver ion gives a greater value for the diffusion current and hence the sensitivity of the determination using $AgNO_3$ is increased.

In order to eliminate the above-mentioned undesirable action of the ammonia buffer solution on the sulfhydryl groups, we decided to use an inverse titration (the solution being studied was added to an excess of $AgNO_3$ solution), in which the SH group was instantly precipitated by silver ion according to the equation:

$$[Ag(NH_3)_2]^+ + RSH \rightarrow RSAg + NH_4^+ + NH_3$$

where RSH is the sulfhydryl compound. The inverse titration is also more suitable because of the small content of SH groups in the water extract of ground-up grain: in direct titration, even the use of 0.001 N $AgNO_3$ would require the use of a large volume of the water extract. It is known that the water extract of ground-up grain contains, in addition to water-soluble proteins, also glucose and fructose, which can reduce silver in ammonia solution. Our experiments showed that: 1) when we added, instead of the water extract of ground-up grain, 1 ml of 0.05 M glucose and fructose solution, under the conditions of our experiment there was no decrease in size of the diffusion current of silver ion; 2) ammoniacal silver solution did not precipitate the proteins of the water extract of grain.

After these preliminary studies we developed the following amperometric method for determining sulfhydryl groups: in a 100 ml glass beaker, closed with a cork with openings for the rotating platinum electrode, microburet, salt bridge to the calomel electrode, and a tube for admitting nitrogen, we placed 50 ml of a solution which contained 0.2 N NH_4OH and 0.2 N NH_4NO_3 (pH 9.37). The solution was freed from oxygen by saturation with nitrogen, which was also passed in during the titration. To this solution was added 1 ml of 0.001 N $AgNO_3$ and at a potential of 0.3 v with respect to the saturated calomel electrode we measured the diffusion current by a mirror galvanometer. Then, through a micropipet we added two or three times 1 ml of the water extract of ground-up grain and observed the value of the diffusion current, decrease in the value of which was directly proportional to the decrease in concentration of silver ions because of their precipitation by SH groups.

For the indicating electrode we used a rotating platinum electrode consisting of a platinum wire 6 mm long with a diameter of 0.5 mm, sealed into a glass tube which rotated at 800 rpm. For the comparison electrode we used a saturated calomel electrode; the salt bridge was filled with 3% agar saturated with NH_4NO_3. For recording the diffusion current we used a mirror galvanometer, type N-21, with a sensitivity of $2 \cdot 10^{-9}$ a/mm/m, reduced 25 times by a direct shunt.

To illustrate the sensitivity of the method we give experimental results: addition to the ammonia buffer solution of 1 ml of 0.001 M $AgNO_3$ causes a deflection in the reflection of the galvanometer of 160 scale divisions; subsequent addition of 0.2 ml of 0.001 M solution of cystine decreases this deflection by 32 scale divisions, which corresponds to 0.2 ml of 0.001 N $AgNO_3$, and addition of 1 ml of water extract of ground-up wheat grains without standing, by 8 scale divisions, which corresponds to

$$8/160 = 0.05 \text{ ml } 0.001 \text{ N } AgNO_3.$$

The concentration of sulfhydryl groups was determined in autolyzed mash from ground-up grain. We

studied the mash from wheat, rye and barley without standing, and also after two and four-hour standing, since the initial stages of proteolysis are the most important for the technology of baking bread. The types of grain from the 1956 crop were: Lyutestsens 17 winter wheat, Petkusskaya rye, and Izariya barley, grown at L'vov. They satisfied the requirements for grains established by GOST.

For an investigation of deep proteolysis we also titrated protein-free filtrates from water extracts obtained by precipitation of the proteins with 2% trichloroacetic acid. The water extracts and protein-free filtrates were obtained by the method described in detail earlier [15].

It is known that the proteolytic activity of grains is sharply increased when they sprout. In order to follow the changes in content of SH groups, the grains were sprouted in the dark for 96 hours (from beginning of soaking) at 16°, then dried in a thermostat at 35° for 12 hours and studied by the method described above.

The results of the determination (average values for three parallel, well-reproducible experiments) are given in the Table.

Sulfhydryl Group Content in Water Extracts and Protein-Free Filtrates of Ground Grains in Autolysis (in μg-equiv per g absolutely dry grain)

Object studied	Water extract			Protein-free filtrate		
	length of standing					
	5 min	2 hours	4 hours	5 min	2 hours	4 hours
Wheat	0.5465	0.8125	0.9855	0.5110	0.6570	0.8030
Wheat sprouts	1.4695	2.0075	2.0805	0.8760	1.0950	1.1498
Rye	1.5425	1.7520	1.9710	1.3140	1.6790	1.8980
Rye sprouts	2.3725	2.4455	2.5550	2.1170	2.2630	2.4820
Barley	0.8395	1.1315	1.2045	0.6570	0.7300	0.7665
Barley sprouts	1.5330	1.6060	1.6425	1.2460	1.3870	1.4600

The data of the Table show that in autolysis the content of SH groups changes differently for different cereals.

Comparing the results for normal and sprouted grains, we see that even without standing there is a sharp increase in content of SH groups in sprouted grain. These results agree well with the literature data on the increase in proteolytic activity in sprouting grain.

Finally, when we compare the results for water extracts and protein free filtrates, we see that there are different depths of proteolysis for normal and sprouted grains of different cereals. We can conclude that in normal wheat grains the depth of proteolysis is less than in rye or barley. With sprouting, the depth of proteolysis is considerably increased, which agrees well with the results for poorer quality of gluten.

SUMMARY

The method of amperometric titration of SH groups by silver nitrate in ammoniacal medium with a rotating platinum electrode has given a determination of the content of SH groups in water extracts of ground-up grain without standing and also after two and four-hour autolysis. In order to preserve the SH groups from oxidation and partial binding by ammonium ions at relatively high pH values for an ammonia buffer, we have used inverse titration. We have studied water extracts and protein-free filtrates of wheat, rye and barley (normal and sprouted for 96 hours). We have shown that in autolysis for four hours, the content of SH groups increases in all cases (much more strongly in sprouted grains).

LITERATURE CITED

[1] G. Shamshikova, Uchenye Zapiski MGU, Botanika 36, 12 (1940).

[2] A.N. Sutulov, Doklady Akad. Nauk SSSR 53, 355 (1946).

[3] A.K. Moskaleva, The Splitting of Sulfur-Containing Amino Acids in Proteolysis. Dissertation [in Russian] (Moscow, 1948).

[4] N.I. Proskuryakov, Sb. Biokhimiya Khlebopecheniya 1, 61 (Izd. AN SSSR, Moscow, 1938).

[5] V.L. Kretovich, A.A. Bundel and T.V. Drozdova, Biokhimiya 13, 332 (1948).

[6] P. Nordin and E.V. Spencer, Cereal Chem. 29, 29 (1952).

[7] I. Kolthoff and J. Linheim, Polarography [Russian translation] (Goskhimizdat, Moscow-Leningrad, 1948).

[8] P. De Large and H.M.R. Hintzer, Cereal Chem. 32, 307 (1955).

[9] J.M. Kolthoff, W. Stricks and L. Morren, Analyt. Chem. 26, 336 (1954).

[10] H. Matsumoto, Fermentation Technol. 33, 233 (1955).

[11] R.E. Benesch, H.A. Lardy and R.J. Benesch, J. Biol. Chem. 216, 663 (1955).

[12] R.W. Kong, D.K. Mecham and I.W. Pence, Cereal Chem. 34, 207 (1957).

[13] D.E. Smith, I.P. van Buren and I.S. Andrews, Cereal Chem. 34, 337 (1957).

[14] J.M. Kolthoff and W.E. Harris, Ind. Eng. Chem. (Anal. Ed.) 18, 161 (1946).

[15] G.I. Kotlyar, Biokhimiya 18, 423 (1953).

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THE INTERRELATION OF PHOTOSYNTHESIS AND RESPIRATION IN PLANTS

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In recent decades a number of facts have been established which demonstrate the participation in photosynthesis of many of the intermediate products and enzymes which take part in the process of respiration [1-3]. This is the chief reason why the vagueness and contradictions which had characterized our ideas of the relation of photosynthesis and respiration, caused by the great methodological difficulties of studying them, have changed to a conviction of the existence of a close connection between these two important processes in the life activity of plants. However, the nature and mechanism of this relation is still not clear. The only hypothesis which has obtained some prominence and which clearly explains the connection between the photosynthetic cycle and the respiratory cycle of the tricarboxylic acids through the reaction of pyruvic acid with lipoic acid complexes, the hypothesis of Calvin [1], cannot yet be considered sufficiently firmly based or satisfactory since it is fundamentally founded on the fact that the formation in leaves of one of the substances of the respiratory cycle, citric acid, is increased after removing the light.

There is no doubt that the character and degree of the relation of photosynthesis and respiration are reflected in the concentrations of the substances which take part in them and that this can be shown especially clearly in them by the strengthening or weakening of the processes which we are considering.

Hence it is clear that if under different conditions of illumination we consider those rapid changes which should occur in the composition and quantities of a number of products which share in photosynthesis and respiration, then the resulting data would surely explain some of the aspects of the relationship of both processes. With this idea in mind, we carried out experiments using different exposures of leaves in a chamber which contained $C^{14}O_2$ under varying conditions of illumination. The labeled products which were formed were studied by radiochromatographic analysis as previously described [4].

The results of the experiments showed that in photosynthesis, in addition to the sugars, their phosphate esters, and some other substances [1], there was always a sufficiently rapid formation of a number of products which took part in the respiratory cycle: di- and tricarboxylic acids (pyruvic, malic, succinic, oxaloacetic, α-ketoglutaric, citric and fumaric acids) or compounds related to them (alanine, aspartic and glutamic acids). For example, labeled succinic acid was often found even at exposures of 10 sec and malic at exposures of 5 sec.

This, most of all, indicates that when photosynthesis begins, products are formed almost simultaneously which can be used if necessary as substrates for respiration. As to the order of their formation and their quantitative ratio, information on this question is important as an indication of the intensity and direction of the processes which occur. Since labeled products of the respiratory cycle in leaves are formed not only by photosynthesis and transformation of substances in respiration, but also in the reaction of dark absorption of carbon dioxide which always occurs, this situation must be considered in running the experiments.

Analysis of the numerical data given in Table 1 and the radioautographs of the materials permits us to conclude that formation of products of the respiratory cycle, di- and tricarboxylic acids, occurs simultaneously in two directions, but unequally in all the cycles.

In the light it occurs mostly counterclockwise, and in the dark, preferably clockwise.

TABLE 1

Formation of Organic Acids in Leaves Under Different Conditions

Plant	Experimental conditions		Time of exposure, in sec	Radioactivity of acids in 1 g of leaf counts/min		
	regime before exposure	regime of exposure in chamber with $C^{14}O_2$		succinic	malic	citric
Bean	Light 1-2 hours	Light	60	710	1800	160
	" 1-2 "	"	300	4050	2530	390
Tobacco	" 1-2 "	"	60	1100	2570	80
	" 1-2 "	"	300	3480	4990	470
Bean	Dark 12-16 hours	Dark	60	–	220	320
	" 12-16 "	"	300	60	530	1200
Tobacco	" 12-16 "	"	60	40	770	540
	" 12-16 "	"	300	110	3120	2910
Bean	Light 1-2 hours	Dark	60	830	1960	450
	" 1-2 "	"	300	1200	3090	1900
	Dark 12-16 hours	Light	60	120	700	650
	" 12-16 "	"	300	1380	2940	1250

Although photosynthesis, expressed as the total reaction, is formally the opposite of respiration, this is actually not the case. Many reactions which are fully reversible at the same time take definite parts in these two contrary processes, the degree of merging of which is determined by special factors. From this point of view, it is impossible without special study of each reaction to estimate accurately the meaning of all the results given in Table 1. Nonetheless, from an analysis of this table it is possible to observe that photosynthesis and respiration evidently involve the reversible reaction malic acid-succinic acid, since with continuous illumination and with illumination after preliminary darkness, the amount of succinic acid increases regularly with respect to the malic acid, and in the dark period which follows illumination, it decreases.

It is known that the substances which take part in any biochemical process of a cyclic character enter into many reactions outside the given cycle. The unavoidable result is that the transformations of substances in different parts of the cycle occur unequally, and this essentially disturbs the conditions for the reversibility of the process as a whole. Evidently, it is for this reason that there is a weakened uptake of labeled carbon in citric acid in the light, as appears especially in the experiments with tobacco leaves. However, when the leaves are illuminated after a preliminary dark period, the formation of citric acid is strengthened in them. On the other hand, in the light there is a strengthening of the reaction of formation of malic acid by β-carboxylation of pyruvic acid which quickly accumulates during photosynthesis.

As was shown above, the formation of some products of the respiratory cycle in the light or in the dark may be the result of various processes which often occur by the same reaction, but at different rates and in different locations. Also, in one part of the cell, such as in the chloroplasts, because of an excess of reducing substances during photosynthesis, the products which are formed may react predominantly in one direction, and in another part, in another direction. Then the actual migration of substances may strengthen or weaken the same process. For example, if the concentration of malic acid formed in photosynthesis is increased because of migration into one part of the cell, then respiration may be strengthened by its oxidation. However, if in this part of the cell there is also an increased migration of photosynthetic reducing agents which cause transformation of the compound in another direction, respiration may be hindered.

Of great value for the increased interrelationship of photosynthesis and respiration is the migration within the cell of the final products of these processes: carbohydrates, O_2 and CO_2. However, there are almost no data in the literature for the study of this question although it would seem to be easy to investigate. Even when they used the isotope method Brown, Near and van Norman [5], after illuminating a number of plants, did not find a change in absorption of O_2^{18}, which, in agreement with the most prevalent point of view [6], showed that light, as a rule, does not have much effect on intensity of respiration. Other investigators concluded that light greatly

TABLE 2

Use of Evolved $C^{14}O_2$ in Photosynthesis Before Its Excretion From the Leaves

Experimental condition	Radioactivity of leaves in counts/min		Radioactivity of excreted $C^{14}O_2$ in counts/min		% Use of evolved CO_2
	in light	in dark	in light	in dark	
Weak light (~200 lux)	51,650	55,965	543	1337	59.4
Stronger light (~600 lux)	91,762	77,420	707	2074	70.7

hinders or even completely stops respiration in plants, since in their experiments with $C^{14}O_2$ they observed a decrease [7, 8] or complete absence [9] of evolution of CO_2 from the plants in the light. The decisive requirement for the solution of this question is a faultless method to assure that the experiments are carried out under conditions as close as possible to the natural ones. It is clear that decrease or absence of evolution of CO_2 in the light must not be regarded as a hindering of respiration, but as an "uptake" in photosynthesis of the carbon dioxide formed in respiration and not excreted by the leaf. For a quantitative determination of the magnitude of the possible uptake, we carried out the following experiments.

Two-week-old bean sprouts carried on photosynthesis for four hours in the presence of $C^{14}O_2$. After a certain amount of saturation by the isotope, the leaves, not separated from the plants, were placed in a horizontal glass vessel of 30 ml capacity and were sealed in hermetically with a special soft cement (Fig. 1). One leaf was illuminated, one was in the dark. Moist air, deprived of CO_2, was passed rapidly through the vessels (0.8-1.0 liter/min). The CO_2 which was evolved was absorbed as $BaCO_3$, in which the radioactivity was determined. The experiment showed that when CO_2 was lacking in the atmosphere the illuminated leaves evolved less carbon dioxide, since it was taken up in photosynthesis (Table 2).

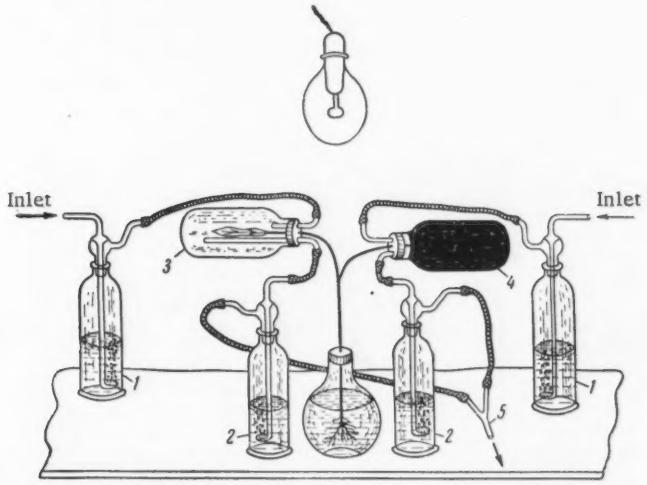


Fig. 1. Plan of apparatus for determining use in photosynthesis of CO_2 evolved by the leaves: 1) CO_2 absorber; 2) $C^{14}O_2$ absorber; 3) light chamber; 4) dark chamber; 5) outlet to water pump,

As would be expected, the degree of this uptake increases with stronger illumination. Since in these experiments there was a high percentage of use of the evolved CO_2 in photosynthesis even at comparatively weak illumination intensity and at the same time with a strong current of air, which could easily remove CO_2 from the intercellular spaces, then under ordinary conditions the utilization can often be still more complete, and can easily reach 100%.

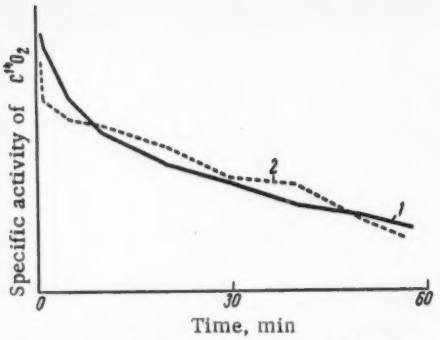


Fig. 2. Specific radioactivity of $C^{14}O_2$ excreted by leaves in the dark after a short period of photosynthesis.

TABLE 3

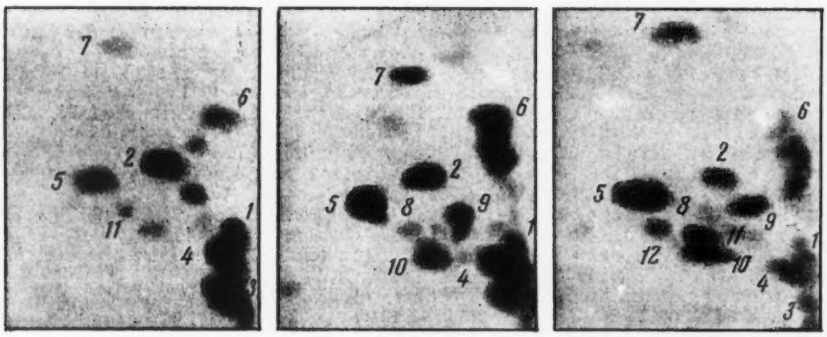
Distribution of C^{14} in Experiments With Bean Leaves in % of Total Radioactivity

Products	After beginning of exposure, in min	
	1	5
Monophosphates	29	5
Sucrose	3	7

In order to solve the question of the degree of probable participation of the intermediate products of photosynthesis in the respiratory process we ran the following experiments.

A normally photosynthesizing leaf was rapidly placed on a special screen which was introduced into a transparent glass chamber containing 0.5% $C^{14}O_2$ in moist air. After 10 sec the leaf was instantaneously transferred under mercury to another darkened chamber in which ordinary CO_2 was present instead of the labeled form. In this preliminary chamber the leaf was, as it were, "washed" free from traces of free $C^{14}O_2$, and after 20 sec it was transferred to a third dark chamber in which it was kept for 30 sec, after which, in a similar way, the leaf was successively transferred at definite times into other dark chambers in accord with the experimental plan. In all these dark chambers, the number of which equaled the number of points taken on the sample, we measured the amount of radioactivity of the evolved $C^{14}O_2$. Since the radioactivity of the leaf and the duration of its exposure in the dark experimental chambers were small, then to get the greatest amount of evolved radioactive $C^{14}O_2$ we had to take a large number of leaves and to repeat the experiment many times.

The determination of the relative radioactivity of $C^{14}O_2$ evolved in equal time intervals showed that carbon dioxide not only began to be evolved in the first minutes after its assimilation, but that the intensity of its excretion at this time was a maximum and close to the value which was obtained if the respiratory requirement of the substrate was supplied by the labeled products which were formed. The specific radioactivity of the excreted $C^{14}O_2$ gradually fell (Fig. 2). Analogous results were obtained by Zalenskii [9] with longer leaf exposures.



A B C

Fig. 3. Radioautographs obtained in experiments with bean leaves: A) 10 sec photosynthesis with $C^{14}O_2$ and 20 sec in dark with CO_2; B) 10 sec photosynthesis with $C^{14}O_2$ and 50 sec in dark with CO_2; C) 10 sec photosynthesis with $C^{14}O_2$ and 290 sec in dark with CO_2. 1) Phosphoglyceric acid; 2) glyceric acid; 3) diphosphate region; 4) monophosphates; 5) alanine; 6) malic acid; 7) succinic acid; 8) serine; 9) aspartic acid; 10) sucrose; 11) glucose; 12) fructose.

It is known [1, 3] that in photosynthesis with short time intervals phosphoric esters of the sugars are chiefly formed: these are intermediate products in the process. Determination of the radioactivity of the products in the leaves at the time of these experiments showed that after 30 sec from the start, the radioactivity was contained mostly in the phosphate esters, and in the sugars there was only a negligible trace. After 60 sec the amount in the free sugars was also slight, and only after 300 sec did they compose a considerable part of the radioactive products (Fig. 3 and Table 3).

It is particularly important to emphasize that in our experiments the respiratory substrates which we studied are not substances introduced by any process from outside, but are metabolites formed in the leaves under natural conditions. We see that the intermediate products of photosynthesis formed under these conditions can be used intensively, exactly there, during respiration, and probably enter the chloroplasts. These results to some extent agree with the conclusion that in the light, plants usually do not use those carbohydrates which are the end product of photosynthesis, for respiration [10].

Thus we have shown in different ways the close connection of photosynthesis and respiration and their union in the total metabolic process. In view of the characteristics of metabolism in different types of plants even when there are many common traits in the interrelation of both processes, there are definite differences in the way these interrelations are shown, which often lead to contradictory conclusions in such major questions as whether respiration is increased or suppressed in photosynthesis. If we consider the processes of building up and breaking down as a unit, we must admit, within the limits of normal life activity, that it is necessary to strengthen one process when the other is intensified. However, this intensification is very irregular for different bonds, and is expressed qualitatively in different ways, which determine the shift in the general direction of metabolic processes. We must remember that the respiratory process can go by different paths [11] with preferential use of the one most adapted to the conditions of the system [12] up to the system of anaerobic respiration.

We therefore think that the interrelation of photosynthesis and respiration is not limited by the forms which are now known. The recently established fact [13] that glycolic acid, formed in photosynthesis, shares in the cycle of transformations of dicarboxylic and tricarboxylic acids confirms [14] the earlier assumption of the possibility of the interrelation of these processes by this channel also.

It is certain that a further understanding of the chemistry of photosynthesis and respiration will show their still closer relationship.

SUMMARY

We have determined the change in composition and amount of a number of products which are formed in leaves when they are exposed in a chamber, in the presence of $C^{14}O_2$, in the light or the dark, and which share in both photosynthesis and respiration; on this basis we have explained the different aspects of the interrelations of the two processes. We have shown that under natural conditions the intermediate products of photosynthesis can be used intensively in the respiratory process, supplying the chief substrate requirements of respiration in the light.

LITERATURE CITED

[1] M. Calvin, Rapports et conferences de 3-ème congres du biochimie (Liège, 1956).

[2] S. Ochoa and W. Vishniac, Science 115, 297 (1952).

[3] N.G. Doman, Reports of the Second All-Union Conference on Photosynthesis, Jan. 26, 1957 [in Russian] (Izd. AN SSSR, Moscow, 1959) (in press).

[4] N.G. Doman, Proc. Commission on Analytical Chemistry, vol. VI [in Russian] (Izd. AN SSSR, Moscow, 1955) p. 452.

[5] Cited by H. Gaffron and E.W. Fager, Ann. Rev. Plant Physiol. 2, 87 (1951).

[6] E. Rabinowitch, Photosynthesis, vol. I [Russian translation] (IL, Moscow, 1951).

[7] J.W. Weigl, P.M. Warrington and M. Calvin, J. Am. Chem. Soc. 73, 5058 (1951).

[8] E. Steemann-Nielson, Physiol. plantarum 8, 945 (1955).

[9] O.V. Zalenskii, Reports of the Second All-Union Conference on Photosynthesis, Jan. 26, 1957 (Izd. AN SSSR, Moscow, 1959) (in press).

[10] P.V. Vittorio, V.G. Krotko and G.B. Reed, Canad. J. Bot. 32, 369 (1954).

[11] D.M. Mikhlin and P.N. Kolesnikov, Biokhimiya 12, 452 (1947).

[12] B.A. Rubin, E.V. Artsikhovskaya, V.E. Sokolova and T.M. Ivanova, Doklady Akad. Nauk SSSR 85, 1123 (1952).

[13] H.L. Kornberg and H.A. Krebs, Nature 179, 988 (1957).

[14] P.A. Kolesnikov, Biokhimiya 13, 370 (1948).

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THE METHOD OF CHROMATOGRAPHY OF SOME PHOSPHATE ESTERS OF SUGARS

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In addition to other methods, we have used the method of chromatography in the study of the transformations of the phosphates of fructose (fructose-1,6-diphosphate and others). In the general literature on this question we have found a number of very contradictory statements (for example, on the rate of migration of fructose phosphate) and poorly worked out methods (use of an insufficiently specific developer, etc.) which impelled us to investigate some problems in the chromatography of sugar phosphates, with especial attention to fructose phosphates.

Rate of Migration of Fructose Phosphates in Chromatographic Separations

Since specific developers for fructose phosphates have not been available up to now, the phosphate groups which they contain have been used to detect them, and so the rate of migration of the phosphates, as determined by the spots on the chromatogram, becomes very important. Many authors have used, for the chromatography of sugar phosphates, systems of similar character which contain neutral organic solvents (methanol, propanol, butanol, etc.) and organic acids (acetic, formic, etc.), and have obtained for the fructose phosphates not only different R_f values, but in a number of cases, mutually contradictory positions of the spots (see the Table).

Thus, of the three substances, inorganic phosphate, fructose-6-phosphate (F-6-P), and fructose-1,6-diphosphate (F-1,6-P), according to the data of Benson [1], the most rapidly moving is orthophosphate, the least so, F-1,6-P, and F-6-P occupies an intermediate position. Analogous results were obtained by Wade [2].

According to the data of Caldwell [3] and Hanes and Isherwood [4] in some analogous acid solvents F-6-P and F-1,6-P move at the same rate (have the same R_f). Finally, Burrows [5] and Bandurskii and Axelrod [6], contradicting Benson [1], found that F-1,6-P in an analogous acid system moves faster than F-6-P.

It is quite evident that even small changes in the relative amounts of solvent in the system and the replacement of one or the other of the neutral and acid components can greatly change the R_f of the chromatographed compounds. However, several cases are known in which there is some change in the absolute value of R_f but the order of succession of the spots, from substances which are close to each other, is still maintained in sufficiently different solvents of the same type.

It is known that in the chromatography of adenine coenzymes the rate of migration is AMP > ADP > ATP and this order is maintained in all acid solvents used: n-butyric acid + sodium butyrate, pH 3.5 [2], tert-butanol + picric acid + water, acetone + trichloroacetic acid [5], acetone + monochloroacetic acid [5], acetone + formic acid [5], n-propanol + acetate buffer, pH 4 [7], isobutyric acid [8], n-butanol + acetic acid [9]. An analogous effect, the slower migration of compounds with a greater number of phosphate residues, was observed also for inosinic compounds: the rate of migration of inosine-5'-monophosphate > inosine-5'-diphosphate > inosine-5-triphosphate, and also for uridine compounds: uridine-5-phosphate > uridine-5-triphosphate [2]. Finally, similar results are also known for a series of sugar phosphates: the rate of migration of glucose-6-phosphate > glucose-1,6-diphosphate; ribulose-5-phosphate > ribulose-1,5-diphosphate; sedoheptulose-7-phosphate > sedoheptulose-1,7-diphosphate [10].

On the basis of all these results we can expect that F-6-P should migrate more rapidly in a series of acid

solvents than F-1,6-P. The data on the more rapid migration of F-1,6-P [5, 6] and also on the same rate of migration of the mono- and diphosphates obtained by some authors [3, 4] can be explained by methodological errors such as insufficiently prolonged experiments, insufficient identification of the spots or some similar fact (effect of contaminants, etc.).

A detailed survey of the literature shows that actually in those studies in which the migration of diphosphate was found to be slower [1, 2] the investigation was carried out much more carefully. Thus, Benson [1] identified F-6-P and F-1,6-P by removal of the "spots" and determination of the ratio of fructose and phosphoric acid in them.

In distinction to this, in the study [6] in which more rapid migration of F-1,6-P was found, identification of the spots was carried out only by development with the Hanes-Isherwood reagent ($HClO_4$, NH_4MoO_4, HCl), which hydrolyzes phosphate esters and develops only the phosphoric acid.

Although these facts indicate greater reliability for the studies [1, 2] than for [5, 6], only experiments can give a final solution to the problem.

To test the results quoted above, we ran several sets of experiments in which we chromatographed inorganic phosphate, F-6-P, and F-1,6-P in different acid solvents which were used by the authors mentioned above (see the Table) and also several other systems.

The sodium salt of F-1,6-P which we used in our work was obtained by the method of Stepanenko and Bobrova as a stable, sterile preparation [11]. Such a preparation, judged by the content of organic phosphorus and fructose, contained more than 90% F-1,6-P (based on dry weight). As the chromatographic experiments showed, the contaminants were inorganic phosphate and traces of 3-phosphoglyceric acid (the sample of the latter was prepared according to [12]). In some of the experiments the sodium F-1,6-P was submitted to more severe conditions (while seeking a method of sterilizing the sample) and F-6-P was also present.

The pure preparation of F-6-P needed for the chromatogram was obtained by hydrolysis of the barium salt of F-1,6-P in N HBr [13]. A 1% solution of the sodium salt was prepared by dissolving the barium salt and precipitating the Ba with a Na_2SO_4 solution.

Generally, we used the ordinary methods of ascending and descending chromatography, but when we tested the data of Ganguli [14] we used the method of radial chromatography of Giri and Rao [15]. Chromatography was carried out on Leningrad paper.

Identification of the spots on the chromatogram was carried out in different ways.

1. Development of the chromatogram according to Wade and Morgan [16] without preliminary hydrolysis of the ester by ferric chloride and sulfosalicylic acid. In this method the inorganic phosphate, and also the easily and difficultly hydrolyzed phosphate esters, are developed as white spots on a rose-lilac ground. This method gives a chromatogram which can be used for further identification of the phosphate ester spots, for example, by establishing the nature of the sugar component.

2. When there was any difficulty in identifying the F-1,6-P spot, we used the following procedure: on a number of sheets of paper we deposited spots of the mixture being studied and also samples of it which had been hydrolyzed in N HCl for various time intervals (usually 1, 2, 3 and 4 days) at 37°. Since under these conditions there was a gradual splitting of the phosphate groups in position 1, we observed, in the following neutralized (NaOH to pH 7.8) solutions and developed chromatograms, a gradual decrease in the spots for F-1,6-P and an increase in the spots for F-6-P and inorganic phosphate (see the figure).

3. When we used a specific developer, diphenylamine, it gave different colored spots with F-1,6-P and F-6-P (see page 25).

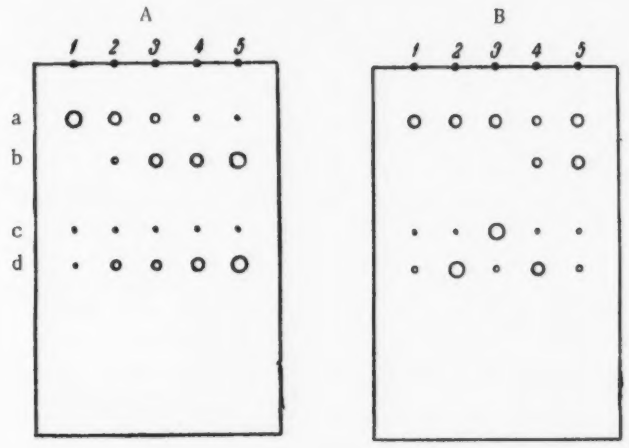
Methods 2 and 3 (especially 3) permitted us to exclude completely the possibility of confusing the spots of F-6-P and F-1,6-P. The experiments which we carried out showed positively that in the nine sufficiently varied systems of acid solvents F-6-P migrates faster than F-1,6-P (see Table).

As to some of the literature data on the more rapid migration of F-1,6-P compared to F-6-P [5, 6], and also the slower migration of inorganic phosphate compared to F-6-P [14], we suggest that in these cases there was an error in the identification of the spots produced by the Hanes-Isherwood reagent in hydrolyzing to inorganic

phosphate [14] or else a strong effect of contaminants. Our data, obtained by the two methods described above (gradual hydrolysis of F-1,6-P and development with specific developers which give different colors with fructose mono- and diphosphates), leave no doubt that in this case F-6-P moves faster than F-1,6-P.

In the Table, like several other authors, we do not always give the R_f values; in a number of cases, especially when we use rapidly flowing solvents, we limit ourselves only to recording the order of distribution of the spots. The rate of migration of the different compounds, as is known, depends on the nature of the substance and the system of solvents, and also on many other factors, paper, temperature, etc., and so we agree completely with a number of authors [1, 6, 14] who consider that we cannot always hope for complete agreement of R_f.

A somewhat rougher, but still more reliable (in the reproducible sense) characteristic of these substances is the order of distribution of their spots.



Plan of the chromatogram obtained in the identification of F-1,6-P by gradual hydrolysis (A), and also in the identification of contaminants in the preparation of sodium F-1,6-P (B). A: 1) Initial 1% solution of F-1,6-P; 2-5) solutions previously hydrolyzed for 1, 2, 3 and 4 days at 37°. Shows decrease in first series spots, a – F-1,6-P, and increase in second series spots, b – F-6-P, and in later series, d – inorganic phosphate. The chromatogram ran for 49 hours in the system n-butanol-acetic acid-water (77 : 19 : 50); developer $FeCl_3$ and sulfosalicylic acid, and then diphenylamine. After the first developer, all the spots were white, after the second, the spots of series a were rose, series b, blue-violet, white spots for series c (phosphoglyceric acid), and spots d disappeared. B: 1) 1% solution of sodium F-1,6-P; 2) F-1,6-P + inorganic phosphate; 3) F-1,6-P + phosphoglyceric acid; 4) partly hydrolyzed solution of sodium F-1,6-P; 5) F-1,6-P + F-6-P. Chromatographed, treated, and developed as in chromatogram A.

Effect of contaminating substances on rate of migration of phosphate esters. The fact of an effect by contaminants on the migration of substances in chromatography has long been well known. Aside from general discussions in handbooks on chromatography, there have recently been given in the literature several concrete examples of the effects of various substances on the distribution of different groups of compounds. Thus, according to the results of Benson [1], inorganic phosphate slows the migration of phosphate esters. Doman and Kagan [17] observed that inorganic phosphate and glucose-1-phosphate mixed with other esters move faster in the system methanol-formic acid than in the pure state.

Results of Chromatography of Fructose Phosphates and Orthophosphates According to Our Data (A) and the Literature Data (B)

Solvent systems (volume in ml)	Position of spots from starting point and R_f*						
	A			B			
	F-1,6-P	F-6-P	P**	F-1,6-P	F-6-P	P**	Literature reference
N C_4H_9OH 74 CH_3COOH 19 H_2O 50	I	II	III	I	II	III	[1]
N C_2H-COOH C_2H_7COONa pH 3,5	—	—	—	I (0,12)	II (0,24)	III (0,36)	[2]
$(CH_3)_3COH$ 80 $(NO_2)_3C_6H_2OH$ 4 H_2O 20	I	II	III	I (0,67)	I (0,67)	II (1,0)	[3]
The same	I	II	III	I	I	II	[4]
$(CH_3)_2CO$ 60 HCOOH 35% 40	I (0,31)	II (0,41)	III	II (0,42)	I (0,40)	III	[5]
$(CH_3)_2CO$ 70 $CClH_2COOH$ 50%	I	II	III	II (0,26)	I (0,15)	III (0,58	[5]
CH_3OH 80 HCOOH 88% 15 H_2O	I (0,45)	II (0,49)	III	II (0,40)	I (0,34)	III	[6]
N C_4H_9OH 4 CH_3COOH 1 H_2O 5	I (0,16)	II (0,49)	III(1,0)	—	—	—	—
The same	I	II	III	—	II	I	[14]***
C_2H_5OH 70 CH_3COONH_4 pH 3,8 35	I	II	—	—	—	—	—
C_2H_5OH 70 HCOOH 1 H_2O 29	I	II	—	—	—	—	—

*Values of R_f enclosed in parentheses.

**P – orthophosphate.

***Radial chromatography.

We observed analogous effects in the chromatography of sugar phosphates in several systems of acid solvents.

When we carried out experiments in the solvent n-butanol-acetic acid-water (74 : 19 : 50) and n-butanol-propionic acid-water (100 : 50 : 70) we found in repeated experiments on untreated paper that, like glucose-1-phosphate, F-6-P moved more slowly in the pure state (as an indicator) than in a mixture with F-1,6-P. However, we cannot conclude from this that it is impossible to obtain accurate results in chromatography, but, as we think, these data give on the one hand a new illustration of the need for more careful measurement of R_f values for the identification of substances in mixtures, and on the other, the accumulation of such evidence can perhaps lead to future introduction of corrections in the results of chromatographic experiments which are run in complex mixtures in accordance with the nature of the substances which are the contaminants.

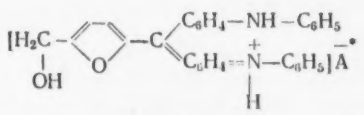
Specific Developers Which Permit Separation of F-6-P and F-1,6-P

The change in rate of migration of the sugar phosphates, which was mentioned above, depends on many

factors, partly on contaminants and on the possibility of errors from using nonspecific developers, thus it is desirable to use specific developers.

Since we wished to find the developer which would give the easiest separation of F-6-P and F-1,6-P, we made many experiments and at last found that such a developer could be diphenylamine. This, as we showed, gives a blue-violet color in the presence of F-6-P, and a rose color in the presence of F-1,6-P.

The colors which occur when fructose reacts with diphenylamine were described by Ihl [18] more than half a century ago. Dische [19] used this reaction for the colorimetric determination of fructose. Liesendahl and Schreier [20] used diphenylamine in paper chromatography of free ketoses. According to the investigations of Kallinich [21], at the basis of this reaction lies the transformation of fructose into hydroxymethylfurfural and the condensation of the latter with diphenylamine to form a dye of the triphenylmethane type:



As is known, phosphate groups in position 1 of F-1,6-P are hydrolyzed much more easily than those in position 6. When the chromatogram moistened with the acid developer is heated, there is certainly splitting of the phosphate in position 1, as is indicated by the appearance of a color with diphenylamine (without splitting of this phosphate group an aldehyde group could not form hydroxymethylfurfural or its 6-phosphate to react with formation of dye).

The change in color of the triphenylmethane dye under the influence of acid, usually with a shift to the long-wavelength part of the visible spectrum, occurs because of formation of a salt with two or three equivalents of acid which reacts with the amino groups in the molecule; this is well known. Evidently, in the present case, the hydrolysis of the ester leads to the formation of a divalent dye ion of a rose color.

This is confirmed by the following experiment: if to the solution of hydroxymethylfurfural formed by boiling a solution of fructose with 7% HCl is added a developer which contains diphenylamine and it is heated, there is formed a blue color; addition of a small amount of H_2SO_4 or H_3PO_4 turns this blue color to rose.

Method of developing the fructose esters. For the preparation of the developer 1 g of diphenylamine was dissolved in 1.5 ml of concentrated H_2SO_4 and glacial acetic acid was added to a volume of 100 ml. The dried chromatogram was sprayed with this developer and heated in a drying oven 5 min at 80-90°. The spot of free fructose and F-6-P became blue-violet while the spot for F-1,6-P became rose. It was impossible to confuse the free fructose and the F-6-P, since free fructose moves much faster than all the sugar phosphates and inorganic phosphate. Thus, for example, in the system with n-propanol and acetate buffer, pH 3.8 (70:30) at room temperature after 15 hours gave R_f based on the migration of inorganic phosphate, that is, R_{fi}; we found for F-1,6-P 0.18, for F-6-P 0.8, inorganic phosphate 1.0, and for free fructose, 2.85.

The sensitivity of this method is 16-20 $\mu g/cm^2$ for F-1,6-P and 6-12 $\mu g/cm^2$ for F-6-P.

Experiments run with synthetic F-1-P showed that this ester, as would be expected, like F-1,6-P, gives a rose color; the spot for F-1-P in chromatography in the solvents n-butanol-acetic acid-water [1] and n-propanol-acetate buffer, pH 4.0 [7] is close to the spot for F-6-P, but differs sharply from it in color.

The Effect of Sugar Concentrations on Their Migration on the Chromatogram

At the very beginning of our work on the chromatography of the phosphate esters we encountered the following phenomenon: when the chromatographic paper was washed with 0.1 N HCl and then with water and dried, and then when 40 μg of F-1,6-P was placed on it with subsequent separation in the system n-butanol-acetic acid-water (74:19:50) and development, we observed two spots of nearly the same intensity. On the unwashed paper under the same conditions this effect was not found; only one spot was obtained.

The first assumption, that on the washed paper there occurred a separation of different substances (fructose diphosphate and its contaminants), was not supported by the facts. Both spots after development with ferric

•$\overline{A}$ – acid anion.

chloride and sulfosalicylic acid showed the presence of phosphoric acid, that is, appeared white on a violet-rose ground; when this chromatogram was then developed by the above-described diphenylamine method the spots became rose colored, which showed the presence of fructose diphosphate. The development of these chromatograms by a freshly prepared solution of phloroglucinol (25 ml of glacial acetic acid, 1 ml of HCl, and 2.5 ml of a 5% solution of phloroglucinol in ethanol) gave an orange-brown colored spot, which also indicates the presence of F-1,6-P and excludes the presence of ribulose phosphate (which gives a dark green color) and sedoheptulose (which gives a green color) [22]. Thus, the separation of F-1,6-P on paper washed by HCl into two spots can be considered to be established.

There is much interest in explaining the question of whether the reason for the separation of the diphosphate is only the preliminary treatment of the paper, or whether this fact has a more general significance, that is, the separation of diphosphate can occur also on untreated paper; or whether the separation of the spots is found also in other sugar phosphates. For a solution of these questions we ran experiments with different concentrations of F-1,6-P, F-6-P, and glucose-1-phosphate.

We showed that if the separation into two spots on treatment of the paper with HCl occurred at amounts of 40 μg F-1,6-P, then the same effect could be produced on untreated paper if we used 160 μg of the same ester.

The same results were obtained with F-6-P and glucose-1-phosphate: each of these substances gave one spot on untreated paper at amounts of 20-40 μg ester, and two spots at amounts of 160 μg (development took place as before).

Thus, separation into two spots is a property not only of F-1,6-P but of a series of phosphate esters of sugars. Treatment of the paper with HCl evidently lowers the quantity of ester at which this effect of separation of spots begins; possibly the paper had absorbed some HCl and the later neutral reaction of the wash water in repeated washings is not significant. The following observation also favors the idea of the action of HCl on separation of the spots: when 40 μg of F-1,6-P is chromatographed on paper which was not treated with HCl in the system acetone–acetic acid–water (70 : 15 : 15) with a small amount of HCl (final concentration 0.001 N), the same two spots are formed with different methods of development.

It should be mentioned that the fact of separation of substance into two (or more) spots has been described in the literature with reference to other compounds: Aronoff [23] found a separation of lysine, West and Riley [24] of histamine, and Beck, of glutamic acid [25].

Our results supplement these findings with respect to sugar phosphates. The physicochemical existence of this phenomenon requires special investigation.

SUMMARY

On the basis of experiments on chromatography of fructose esters in nine different acid systems of solvents we have shown that fructose-6-phosphate in all cases migrates faster than fructose-1,6-diphosphate; some descriptions in the literature on the same rate as monophosphates or the faster migration of fructose-1,6-diphosphate under these conditions can be explained by insufficiently prolonged experiments, methodological errors (especially the use of a nonspecific developer) or, probably, by the effect of a complex mixture of contaminating substances.

As a specific developer which can separate the fructose phosphates we have suggested diphenylamine, which gives with fructose-6-phosphate (and also with free fructose) a blue-violet color, and with fructose-1,6-diphosphate and fructose-1-phosphate a rose color. We showed that some sugar phosphates, when added in comparatively large amounts to the chromatogram in definite acid solvents, can give two spots of approximately the same intensity. Treatment of the paper with HCl evidently decreases the amount of ester required to develop this effect.

LITERATURE CITED

[1] A.A. Benson, J. Amer. Chem. Soc. 72, 1710 (1950).

[2] H.E. Wade and D.M. Morgan, Biochem. J. 60, 267 (1955).

[3] P.C. Caldwell, Biochem. J. 55, 458 (1953).

[4] C.S. Hanes and F.A. Isherwood, Nature 164, 1107 (1949).

[5] S. Burrows, F.S.M. Grylls and J.S. Harrison, Nature 170, 800 (1952).

[6] K.S. Bandurski and B. Axelrod, J. Biol. Chem. 193, 405 (1951).

[7] P.C. Caldwell, Biochem. J. 60, 12 (1955).

[8] W. Simonis and L. Schwinck, Naturwiss. 40, 245 (1953).

[9] I.A. Sytinskii, Biokhimiya 21, 358 (1956).

[10] A.A. Benson, Methods in Enzymol. v. 3 (Ed. Colowick and Kaplan, New York) p. 110.

[11] B.N. Stepanenko and L.N. Bobrova, Biokhimiya 22, 1019 (1957).•

[12] V.A. Belitser and M.I. Karlina, Biokhimiya 9, 33 (1944).

[13] C. Neuberg, H. Lustig and M. Rothenberg, Arch. Biochem. 3, 33 (1944).

[14] N.G. Ganguli, Naturwiss. 40, 624 (1953).

[15] K.V. Giri and N.A.N. Rao, J. Indian Inst. Sci. 34, 95 (1952).

[16] H.S. Wade and D.M. Morgan, Nature 171, 529 (1953).

[17] N.G. Doman and Z.S. Kogan, Biokhimiya 17, 719 (1952).

[18] A. Ihl, Chem. Z. 9, 451 (1885).

[19] Z. Dische, Mikrochemie 1, 33 (1929); J. Biol. Chem. 204, 983 (1953).

[20] H. Liesendahl and K. Schreier, Klin. Wochenschr. 33, 590 (1955).

[21] H. This and G. Kallinich, Chem. Ber. 85, 438 (1952).

[22] E. Borenfreund and Z. Dische, Arch. Biochem. and Biophys. 67, 239 (1957).

[23] S. Aronoff, Science 110, 590 (1949).

[24] G.B. West and T.F. Riley, Nature 174, 882 (1954).

[25] M.T. Beck and P. Ebrey, Biochim. et biophys. acta 20, 393 (1956).

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A COMPARATIVE STUDY OF SOLUBLE COLLAGEN-LIKE PROTEINS

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At the present time, on the basis of much experimental data, we can say that there exist in the skin and some other organs two forms of protein of the collagen type: one soluble in the usual protein solvents, procollagen, and the other insoluble, collagen itself.

The interest of investigators in the soluble form of collagen was especially aroused when it was isolated in the pure form as a collagen-like protein (procollagen) and its biological and chemical properties were studied [1]. In recent times a number of methods have been described for isolating the soluble collagen-like proteins from connective tissue of different animals [2, 3]. Depending on the method of obtaining these proteins, they are called acetate-soluble proteins (ASP), neutral-soluble, citrate-soluble proteins (CSP), and alkali-soluble collagens. The alkali-soluble collagen in the opinion of some English authors is the true precursor of collagen [4], although according to the data of other authors [5, 6] it does not differ in its chemical and physicochemical properties from citrate-soluble collagen. In its turn, neutral-soluble collagen has a great similarity to alkali-soluble and so there is reason to consider all three proteins identical [6]. Special study of the comparison of properties of ASP with the other soluble collagen-like proteins has not been made.

We therefore felt it would be interesting to study some chemical, physicochemical and immunobiological properties of collagen-like proteins dissolved in dilute acetic acid and to compare these with the analogous properties of the citrate-soluble proteins.

EXPERIMENTAL

The skin of white rats, freed from hair, epidermis, and subcutaneous fat tissue, was washed with water and cut up with scissors. The same weight of cut-up skin was treated in one case with ten times the volume of 0.1 M solution of a citrate buffer, pH 3.8-3.9, and in another with ten times the volume of 0.01 M solution of acetic acid. Extraction was carried out at 4° for 24 hours. When we had obtained nine daily extracts of ASP and CSP, we replaced one solvent by the other and carried out further extractions for the following nine days. Thus, the total duration of extraction was 18 days. The 24-hour extracts of ASP and CSP, after filtration through a paper filter, were precipitated with an equal volume of 10% NaCl. The protein precipitate was separated by centrifuging, washed with 0.3 M Na_2HPO_4 solution, then several times with tap water, and twice with distilled water. The protein was dried with 96% alcohol, a mixture of alcohol and ether (1 : 1), and ether, and was brought to constant weight at 100-110°. The concentrations of the proteins in the daily extracts were determined by dry weight and total nitrogen. The amount of protein determined by nitrogen was 98-99% of the dry weight of the preparation. The curves of yield of ASP and CSP are given in Fig. 1.

Quantitative determination of proline and hydroxyproline. For the determination we used the protein preparations extracted in the course of 2, 3, 4 and 5 days. Hydrolysis was carried out in 6 N HCl for 24 hours at 100-110°. Hydroxyproline was determined by the method of Neuman and Logan [7] in the modification of Martin and Axelrod [8], and proline, by the method of Troll and Lindsley [9]. The amount of amino acid was expressed in percent of the total dry weight of the preparation. Results are given in Table 1.

Determination of N-terminal amino acids in ASP and CSP was carried out by the method of Sanger [10].

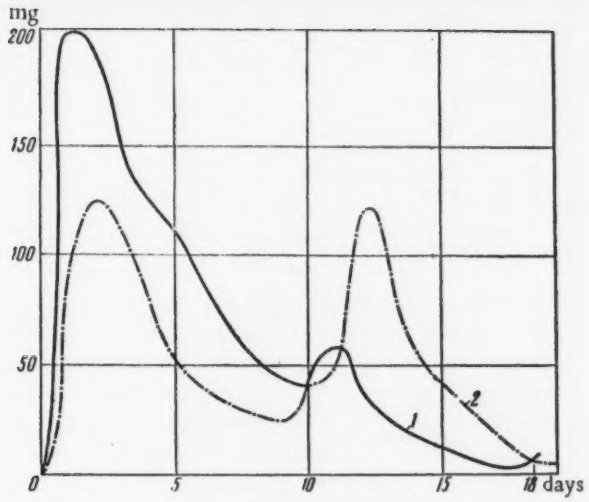

Fig. 1. Curves of yields of acetate and citrate-soluble collagen-like proteins: 1) extracted by citrate buffer; 2) extracted by acetic acid. Exchange of one solvent for the other was carried out after nine days extraction.

TABLE 1

Content of Hydroxyproline and Proline in Daily Extracts of ASP and CSP

Amino acid	2 days		3 days		4 days		5 days	
	ASP	CSP	ASP	CSP	ASP	CSP	ASP	CSP
Hydroxyproline	13.0	13.1	13.8	13.3	13.8	13.3	13.6	13.5
Proline	11.5	12.0	11.0	11.6	12.2	11.0	11.6	10.8

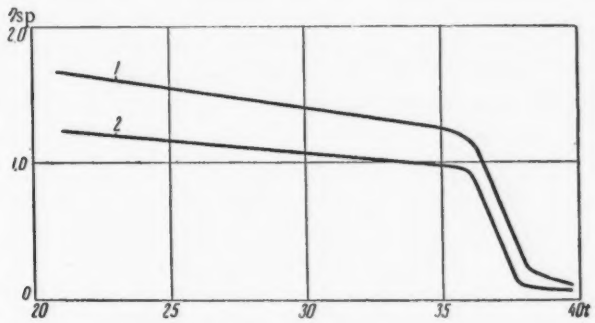

Fig. 2. Relation of specific viscosity to temperature: 1) CSP solution; 2) ASP solution.

One-hundred mg of each of the proteins studied was treated with dinitrofluorobenzene (DNFB) for four hours with constant stirring. Then, after removing the excess DNFB, acid hydrolysis was carried out for 18 hours at 105°. The DNF-amino acids were extracted from the acid hydrolysate by ether and studied chromatographically. We also studied the amino acids which remained in the water fraction. Separation of DNF-amino acids was carried out on paper treated with a phthalate buffer. As the solvent we used a mixture of ethyl and benzyl alcohols (1 : 9)

saturated with phthalate buffer. For quantitative determination, the spots of DNF-amino acids were extracted with 1% $NaHCO_3$ and determined in a spectrophotometer at 360 mμ. On the resulting chromatogram we found weak spots for the derivatives DNF-glycine, DNF-aspartic acid, and DNF-glutamic acid.

Calculation showed that in the molecule of each of the studied proteins (taking the molecular weight as 500,000) there entered 0.01 M of glycine, glutamic, and aspartic acid.

The temperature of viscosity decrease of both proteins was determined in a U-shaped Ostwald capillary viscosimeter at the temperature at which there was a rapid fall in specific viscosity of the two protein solutions. In Fig. 2 we give the graph of relation of specific viscosity (η_{vs}) of solutions of ASP and CSP to temperature. From the course of the curves we can determine that the rapid fall of η_{vs} in both proteins occurs at 37-38°.

Immunobiological study. In the work of Watson, Rothbard, et al. it was shown that collagen has antigen properties [11, 12]. For a study of the immunobiological properties of solutions of collagen-like proteins we used the method of anaphylaxis with desensitization which was worked out in the laboratory of Zil'ber [13]. In preliminary experiments we showed that anaphylactic shock in guinea pigs could be produced a month after injecting 8-10 mg of ASP or CSP. The scheme of running these experiments is shown in Table 2. (For the intravenous injection we used only weakly acid solutions of the protein.)

DISCUSSION OF RESULTS

We showed above that the amount of soluble collagen-like protein could vary depending on experimental conditions. Extraction by one solvent, even if it was carried out for a long time could not guarantee that further amounts of protein might not be removed from the same source under other conditions. In our experiments, after nine-day extraction with citrate buffer or acetic acid, the yield of protein became slight but we could obtain further quantities of protein by exchanging the citrate buffer for acetate and vice versa. When we extracted the rat skin for a longer period (30-40 days) with these solvents we also obtained an increased amount of protein and, in addition, were convinced that long extraction was accompanied by strong swelling and destruction of tissue; along with the collagen-like protein there also occurred solution of a considerable amount of basic mucoprotein substances, polysaccharides, and even separate small pieces of tissue were in suspension. Therefore, analysis of protein preparations obtained by prolonged extraction was difficult and could give incorrect results.

When we carried out an amino acid analysis we considered this fact, and in the experiments we took daily protein extracts during 2-5 days. According to our data, ASP and CSP do not differ from each other in amino acid content. Thus, in ASP there is an average of 13.5% hydroxyproline, and in CSP, 13.3%. The amount of proline in these proteins is 11.6 and 11.1%. Recent literature data has shown that the amino acid composition of soluble collagen-like proteins varies depending on whether the extract of any one day is taken for analysis [14]. In our experiments we did not observe such a relation, since in the protein preparations obtained from the 2, 3, 4 and 5-day extracts, the content of such important amino acids as proline and hydroxyproline was constant. The observed variation evidently lies within the limits of error of the experiment or depends on small mixtures of other substances.

Bowes and Moss, Orekhovich, and other authors determined the N-terminal amino acids in collagen and concluded that these were absent in native collagen preparations [15, 16, 17]. In our experiments on the determination of N-terminal amino acids we obtained the same result for both proteins which we studied: N-terminal amino acids were practically absent in ASP and CSP, since in each case only trace amounts were found in the protein molecule (≈0.01 M) for glycine, aspartic acid and glutamic acid. Since terminal amino acids were found in collagen after treatment with hyaluronidase, periodate, and some other compounds, we can assume that they are blocked by polysaccharides, whose admixtures are found even in highly purified preparations of the protein [18, 19].

When we used the method of anaphylaxis with desensitization in our comparative studies of soluble collagens, we considered that in the preparation of one of the proteins which we have studied some other protein might be present, and this would have a negative effect on the course of the experiment. Nevertheless, when we analyzed the results we found reason to believe that the acetate and citrate-soluble collagen-like proteins were identical (Table 2). Table 2 shows that guinea pigs did not react to the injection of final doses of CSP if they were first desensitized to ASP. Hence, the latter fully eliminates the sensitizing effect of CSP. This indicates that these proteins are identical in immunobiological respects also.

TABLE 2

Characteristics of Acetate and Citrate-Soluble Collagen-Like Proteins by the Method of Anaphylaxis With Desensitization. Designations: − no reaction; + − fall in temperature, sneezing, itching; ++ dishevelled fur, cough, sneezing, strong itching; +++ the same effects, excretion of urine and feces, panting; ++++ convulsions, death from anaphylactic shock. C − control animals

Sensitization			Desensitization (after 30 days)								Final injection		
					intravenous								
					injection I		injection II		injection III				
antigen	animal No.	dose, mg (subcutaneous)	antigen	dose, mg subcutaneous (day before)	dose, mg	reaction	dose, mg	reaction	dose, mg	reaction	antigen	dose, mg	reaction
CSP-collagen	1	8	ASP-collagen	10	2	+++	6	++	9	+−	CSP-collagen	9	+−
	2	8		10	2	+	6	+	9	−		9	−
	3	8		10	2	++	6	+	9	+−		9	−
	4	8		10	2	++	6	++	9	−		9	−
	5	8		10	2	+++	6	+	9	+−		9	−
	6	8										9	++++
Study of toxicity of solution	C				9								−
	C											9	−

Thus, on the basis of the literature and the above-described experimental results it is possible to speak of the identity of the soluble collagen-like proteins in a number of chemical, physicochemical and immunobiological properties and to consider that the so-called soluble collagen or procollagen is the basic material from which collagen fibers are formed [20]. The formation of collagen fibers is a complex process and in it, in addition to aggregation of the separate collagen molecules, a part is played by polysaccharides and mucoproteins which evidently act by binding the separate procollagen molecules, making them less soluble [21, 22]. Recently there have been reports in the literature of the existence in animal connective tissue of a particular protein-polysaccharide complex which has been called metacollagen or collastromin. In the opinion of some authors this complex enters the composition of collagen fibers along with procollagen and determines their morphological and functional identity [23]. In the opinion of others it is a constituent part of collagen, which, considered as a complex system, is composed essentially of two parts: collastromin or metacollagen and procollagen [24, 25]. It seems to us that the idea of metacollagen or collastromin as a component of collagen fibers, which have a complex morphological form, is more probable, which confirms the fact that collagen is a multicomponent system. In the latter case the protein, collagen, is incorrectly identified with the morphological structure, collagen fiber.

SUMMARY

We have studied some chemical, physicochemical and immunobiological properties of collagen-like proteins dissolved in 0.01 M acetic acid solution and in 0.1 M solution of a citrate buffer, pH 3.8-3.9. Quantitative determination of amino acids shows that the content of hydroxyproline and proline in these proteins is the same. Results show that in the acetate and citrate-soluble collagen-like proteins, free α-amino groups are absent. The proteins have the same shrinkage temperature and do not differ from each other in immunobiological properties when studied by the method of anaphylaxis with desensitization. On the basis of this experimental work and the literature data we can consider that all the soluble collagen-like proteins obtained by any process are identical and should be assigned to one group of connective tissue proteins, the procollagens.

LITERATURE CITED

[1] V.N. Orekhovich, A.A. Tustanovskii, K.D. Orekhovich and N.E. Plotnikova, Biokhimiya 13, 1 (1948).

[2] R.D. Harkness, A.M. Marko, H.M. Muir and A. Neuberger, Nature and Structure of Coll. (Butterworth's Scientific Publications, London, 1953) p. 208.

[3] D.S. Jackson and J.H. Fessler, Nature 176, 69 (1955).

[4] R.D. Harkness, A.M. Marko, H.M. Muir and A. Neuberger, Biochem. J. 56, 558 (1954).

[5] V.N. Orekhovich, L.V. Pavlikhina and V.O. Shpikiter, Biokhimiya 22, 210 (1957).*

[6] Chia-Mu Peng and Tien-Chin Tsao, Scientia Sinica 5, 691 (1956).

[7] R.E. Neuman and M. Logan, J. Biol. Chem. 184, 299 (1950).

[8] C.J. Martin and A.E. Axelrod, Proc. Soc. Exptl. Biol. and Med. 83, 461 (1953).

[9] W. Troll and J. Lindsley, J. Biol. Chem. 215, 655 (1955).

[10] F. Sanger, Biochem. J. 39, 507 (1945).

[11] R. Watson and S. Rothbard, J. Exptl. Med. 103, 535 (1956).

[12] R. Watson, S. Rothbard and P. Vanamee, J. Exptl. Med. 99, 535 (1954).

[13] L.A. Zil'ber, Uspekhi sovrem. biol. 27, 185 (1949).

[14] A.A. Denisova and A.L. Zaides, Doklady Akad. Nauk SSSR 114, 1287 (1957).*

[15] J. Bowes and J. Moss, Biochem. J. 55, 735 (1953).

[16] V.N. Orekhovich, Procollagens, Their Chemical Composition, Properties, and Biological Role [in Russian] (Izd. AN SSSR, Moscow, 1952).

*Original Russian pagination. See C.B. translation.

[17] W. Grassman and H. Hörmann, Z. Physiol. Chem. 292, 25 (1953).

[18] W. Grassman and K. Kühn, Z. Physiol. Chem. 301, 1 (1955).

[19] J.H. Bowes, R.G. Elliot and J.H. Moss, Biochem. J. 61, 143 (1955).

[20] D.S. Jackson, Recent Advances in Gelatin and Glue Research (Pergamon Press, London, 1957).

[21] J.A. Bowes, R.G. Elliot and J.A. Moss, Biochem. J. 63, 1P (1956).

[22] D.S. Jackson, Biochem. J. 54, 638 (1953).

[23] J. Banga, J. Baló and D. Szabó, Acta Physiol. Hung. 9, 61 (1956).

[24] A.A. Tustanovskii, A.L. Zaides, G.V. Orlovskaya and A.N. Mikhailov, Doklady Akad. Nauk SSSR 97, 121 (1954).

[25] A.L. Zaides, Biofizika 1, 279 (1956).

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THE EFFECT OF ANAEROBIC CONDITIONS ON CHANGE IN THE RELATIONS OF THE MAIN CAROTENOIDS IN GREEN LEAVES

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In recently published work [1] it was suggested that in the green leaves of plants there is an enzyme system which regulates the relations of the pigments lutein and violaxanthine. Since the violaxanthine molecule contains two epoxide oxygens, it is of interest to explain how this enzyme system acts under completely anaerobic conditions.

OBJECT AND METHODS

The experiments were carried out on leaves of broad beans (Vicia faba L.). Sections from the middle part of the leaves (total weight about 1 g) were placed in tap water in a Bunsen flask with the stoma side up. The flasks were covered with a hood of dark material. A stream of pure hydrogen from a Kipp generator was passed through a system of such flasks for 2-6 hours. For illumination the flasks were detached and transferred to an illumination apparatus. In some variants of the experiments, before the flasks were placed in the light, a stream of hydrogen which contained 5% CO_2 was passed in from a gasometer. Illumination came from below with a 3N-8 lamp of 5000 w. To prevent overheating of the leaf we placed a tap-water screen between the lamp and the object. Illumination in the experiment reached 80-100 thousand lux. In most of the experiments the length of illumination was two hours. After illumination, the leaf section was quickly fixed with cold acetone at −78°. Analyses for the pigments were carried out by the method described earlier [2, 3].

EXPERIMENTAL RESULTS

In the first series of experiments we studied the effect of anaerobic conditions in the dark on the content of lutein and violaxanthine. As the results given in the Table show, anaerobic conditions for six hours in the dark do not affect the percent content or the ratio of lutein and violaxanthine.

Effect of Anaerobic Conditions in the Dark on Content of Lutein and Violaxanthine (in % of total carotenoids)

Conditions	Content in %	
	lutein	violaxanthine
Aerobic	31.2 ± 1.5	24.4 ± 1.4
Anaerobic	33.1 ± 1.0	25.2 ± 2.3

The second series of experiments were carried out to show the effect of light under anaerobic conditions. The results of this series of experiments are given in Fig. 1.

As Fig. 1 shows, in a hydrogen atmosphere without addition of CO_2 the difference in percent content of lutein and violaxanthine increases strongly under the influence of light, reaching 33%. Illumination in an atmosphere of hydrogen with added CO_2 also causes a rise in the size of the difference in percent content of lutein and violaxanthine, though to a lesser extent (to 18.3%). These experiments show that the light reaction can proceed even in the absence of molecular oxygen.

In a third series of experiments we studied the action of darkness after illumination under anaerobic

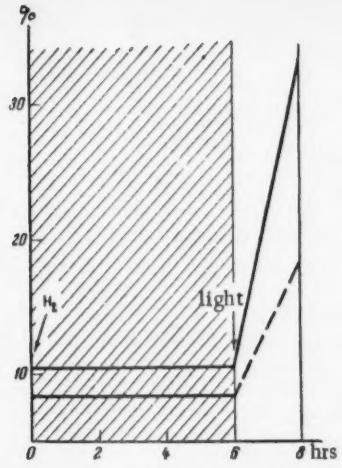

Fig. 1. Action of light on the percent difference in content of lutein and violaxanthine under anaerobic conditions. Shaded area, dark: solid line – atmosphere of pure hydrogen; dashed line – atmosphere containing 5% CO_2 in the hydrogen. On the ordinate is the difference in percent content of lutein and violaxanthine.

conditions. In one of the variants of this series after illumination, the anaerobic state was destroyed by passing oxygen. In Fig. 2 we give the results of this series of experiments.

It follows from curve A that darkness under conditions which are completely anaerobic does not change the level of difference in percent content reached after illumination. In curve B is reflected the result of the experiment in which CO_2 is added to the atmosphere before illumination, and after illumination the plant is maintained in the dark in the same atmosphere. Curve C shows the results of the experiment in which, after illumination in an atmosphere which contains 5% CO_2, oxygen is added. Evidently, the presence of oxygen in the atmosphere lowers the value of the difference in the dark, that is, leads to an increase in the amount of violaxanthine.

In Fig. 2B is shown that the presence of CO_2 in the hydrogen atmosphere even without addition of molecular oxygen can lead to a decreased value for the difference in percent content of lutein and violaxanthine.

DISCUSSION OF RESULTS

As shown in earlier published work [1], when light acts on leaves, there occurs in them a change in ratio of lutein and violaxanthine: the sum of these two xanthophylls remains unchanged while their difference increases in the light and decreases in the dark.

The experiments described in the present work have shown that the increase in this difference under the action of light can also occur

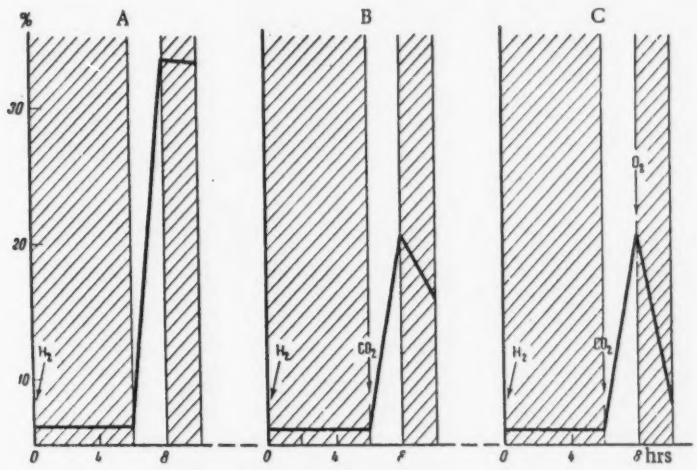

Fig. 2. Action of darkness after illumination under anaerobic conditions on the size of the difference in percent content of lutein and violaxanthine: A) in an atmosphere of pure hydrogen; B) before illumination 5% CO_2 added; C) the same; after illumination, oxygen added.

in the absence of molecular oxygen (in an atmosphere of pure hydrogen). The reverse dark reaction, decrease in the difference in percent content of lutein and violaxanthine, in an atmosphere of pure hydrogen is strongly hindered, which indicates the dependence of this reaction on the presence of molecular oxygen. The addition of molecular oxygen after illumination under anaerobic conditions causes a decrease in this difference in the dark.

In all probability, when the light reaction occurs in an atmosphere of hydrogen, which contains CO_2 the process of photosynthesis can be carried out in the leaf and this in turn destroys the anaerobic condition. This can explain the two facts found in our experiments: 1) less change in ratio of lutein and violaxanthine when light acts under anaerobic conditions in the presence of CO_2 (Fig. 1), and 2) fall in the difference in the dark after illumination without adding molecular oxygen (Fig. 2B).

SUMMARY

In a hydrogen atmosphere in the dark there is no change in the size of the difference in percent content of lutein and violaxanthine. The light reaction, the fall in content of violaxanthine with simultaneous rise in lutein content, proceeds in the absence of molecular oxygen.

Anaerobic conditions hinder the course of the dark reaction – in the absence of molecular oxygen there is no appreciable fall in the difference in percent content of lutein and violaxanthine which is found after illumination. The destruction of anaerobic conditions by introduction of molecular oxygen permits the dark reaction to proceed.

LITERATURE CITED

[1] D.I. Sapozhnikov, T.A. Krasovskaya and A.N. Maevskaya, Doklady Akad. Nauk SSSR 113, 465 (1957).*

[2] D.I. Sapozhnikov, I.A. Bronshtein and T.A. Krasovskaya, Biokhimiya 20, 286 (1955).

[3] D.I. Sapozhnikov, I.A. Bronshtein-Popova, T.A. Krasovskaya and A.N. Maevskaya, Fiziol. rastenii 3, 487 (1956).

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THE DETERMINATION OF DITHIZONE IN ANIMAL BLOOD

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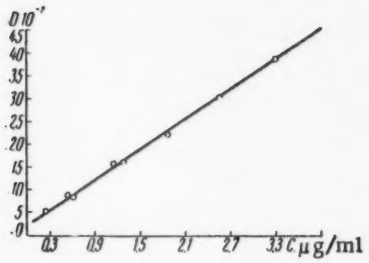
Since dithizone (diphenylthiocarbazone) is at present used in animal experiments to produce diabetes in the subjects, we set ourselves the problem of working out a method for determining dithizone in blood in order to study the dynamics of its transformations in the organism.

Purification of dithizone. Commercial dithizone contains a number of its oxidation products as impurities. The preparation of a standard solution for preparing a calibration curve in its colorimetric determination requires preliminary purification of the preparation. Purification of dithizone was carried out by the method suggested by Babko and Pilipenko [1] with some changes in order to obtain dry dithizone. One-hundred mg of commercial dithizone was dissolved in 50 ml of chloroform in an Erlenmeyer flask with a ground-glass stopper and 50 ml of a 1% NH_4OH solution was added. The solution was extracted 10-15 min on a shaking machine. After the extraction, the aqueous ammonia fraction was separated in a separatory funnel and the chloroform solution was extracted four times more with 25 ml of 1% NH_4OH solution. All the ammonia extracts were combined. It is not recommended to allow the ammonia solution of dithizone to stand, since this permits formation of oxidation products. To the collected ammonia extracts was added a 1% solution (by weight) of H_2SO_4 to an acid reaction. The precipitate of pure dithizone was filtered, washed with distilled water from traces of acid, dried on filter paper and then in a desiccator. A solution in chloroform was prepared for titration from the purified dithizone.



Calibration curve for dithizone.

TABLE 1

Determination of Dithizone in Water Solution

Dithizone content, μg/ml		
Added	Determined	%
429.0	427.0	99.5
459.0	473.0	103.0
128.0	111.0	86.5
15.8	15.8	100.0
8.6	8.2	96.0

Construction of the calibration curve. The solution of dithizone in chloroform was placed in an FM universal photometer. The absorption spectrum of the chloroform solution, taken with all the light filters of this apparatus, showed that the greatest absorption occurred with the light filter λ = 619 mμ. Therefore, all further measurements were carried out with this light filter. The calibration curve was a straight line (see figure).

For the introduction of dithizone into blood we prepared a solution in 0.5% ammonia. The desired amount of dithizone was placed in a flask with a ground-glass stopper and the 0.5% ammonia solution was added so that the amount of dithizone did not exceed 10 mg per 1 ml of solution. The flask was kept in the water bath at 60-70° and frequently shaken during 10 min; then the hot solution was filtered and the warm filtrate was mixed with the blood. As a control, the ammonia solution of dithizone was added to twice-distilled water in the same concentration as in the blood.

In the experiments in vitro dithizone was added to defibrinated blood from cattle; in the experiments in vivo it was injected into the ear or jugular vein of rabbits.

TABLE 2

Determination of Dithizone in Blood

Amount, ml		Amount of added ammonia solution of dithizone, ml		Found dithizone, μg/ml	
H_2O	blood	H_2O	blood	H_2O	blood
100	100	13.3	13.3	427.0	280.0
100	100	13.3	13.3	473.0	368.0
100	100	3.0	3.0	111.0	90.0
100	100	1.0	1.0	35.2	25.5
250	250	1.0	1.0	15.8	11.0
250	250	1.0	1.0	13.3	7.5
250	250	0.5	0.5	8.2	3.5
250	250	0.5	0.5	5.4	2.1
500	500	0.5	0.5	2.4	Impossible to determine

TABLE 3

Content of Dithizone in Water and Blood

Added dithizone, μg/ml	Time of determination	Found dithizone, μg/ml	
		H_2O	blood
429.0	Immediately after addition	427.0	280.0
	After 35 minutes	450.0	286.0
	After 2 hours 30 minutes	427.0	286.0
459.0	Immediately after addition	473.0	368.0
	After 50 minutes	450.0	368.0
	After 1 hour 45 minutes	450.0	363.0
128.0	Immediately after addition	111.0	90.0
	After 30 minutes	111.0	100.0
	After 2 hours 35 minutes	–	87.0
41.2	Immediately after addition	35.2	25.5
	After 30 minutes	–	25.6
8.6	Immediately after addition	8.2	3.5
	After 30 minutes	–	3.3
–	Immediately after addition	5.4	2.1
	After 45 minutes	5.4	2.0

Method of determining dithizone. An exactly measured volume of ammonia solution of dithizone was added to a definite volume of defibrinated blood in a calibrated flask with a ground-glass stopper, first heated in a thermostat to 37°. The mixture was carefully shaken and at once 2 ml of blood was measured out in a thick-walled tube with a flat ground-glass stopper, to which had first been added 5 ml of chloroform. The tube was fixed in an apparatus for shaking in a horizontal position parallel to the bottom with 130 oscillations per minute. Simultaneously we set up an experiment in which instead of defibrinated blood we used twice-distilled water. The experiment with the water was carried out in exactly the same way as with the blood. Extraction lasted 15 minutes. The contents of the flask were poured quantitatively into a separatory funnel. The chloroform extract was quantitatively separated from the blood (water) into a calibrated flask. To test the completeness of removal of the dithizone from the residual blood (water) another 15-minute extraction with chloroform was carried out.

In most cases with repeated extraction the chloroform was not colored, but rarely there was a slight greenish tint to the chloroform extract. In this case it was again separated quantitatively from the blood (water) and combined with the first extract; then all was diluted to the mark with chloroform and determined colorimetrically. The dithizone concentration was established from the calibration curve.

We showed that dithizone added to twice-distilled water was removed from it practically completely by chloroform (Table 1).

Dithizone added to blood in the same concentration as to water always showed a smaller amount than in water (Table 2). This was because after addition of dithizone to blood there was partial hemolysis of the erythrocytes which was stronger, the greater the added solution of dithizone.

The zinc which is liberated from the erythrocytes combines with the dithizone [2]. The dithizone which is not combined with the zinc of the erythrocytes is determined completely. To show this, we determined the content of dithizone in blood and in water with time. The flask with the blood which contained dithizone was placed in a thermostat at 37°. At equal time intervals we measured out a definite amount of blood and determined the dithizone in it. At the same time we also studied the twice-distilled water. The results are given in Table 3. The data of this table indicate that we have actually determined the absolute amount of dithizone in blood, since with repeated determinations at equal time intervals we obtain identical results.

This method for the determination of dithizone in blood was used for measuring the rate of binding of dithizone in the organism. Rabbits were injected intravenously with different amounts of dithizone and then after 3-20 minutes the dithizone content of the blood was determined. The results are given in Table 4.

TABLE 4

Dithizone Content in Blood After Intravenous Injection in Rabbits

Weight of rabbit, g	Injected dithizone, μg/kg	Dithizone found in μg/ml of blood after			
		3 min	5 min	10 min	20 min
1420.0	40,000	18.6	13.5	9.8	5.2
1130.0	40,000	19.5	13.8	9.0	3.3
1350.0	40,000	24.9	6.3	2.6	None
1400.0	56,000	–	6.15	2.8	1.2
1300.0	52,000	39.9	10.2	4.5	3.0

It was shown that injection of large amounts of dithizone (40,000-56,000 μg) resulted in its very rapid disappearance from the blood. Twenty minutes after its injection into blood, only 1.2-5.2 μg/ml were found. Evidently, in organs and tissues there is rapid formation of metal dithizones which may be the reason for the various pathological processes (diabetes, etc.).

The study of the transformations of dithizone in the organism will be the subject of our future investigations.

SUMMARY

We have described a method for determination of dithizone in blood. The sensitivity of the determination is 0.15 μg/ml.

When large amounts of dithizone (40,000-56,000 μg) are injected into the blood, they disappear unusually rapidly. Within 20 minutes after the injection the dithizone content of the blood becomes negligible.

LITERATURE CITED

[1] A.K. Babko and A.T. Pilipenko, Colorimetric Analysis [in Russian] (Goskhimizdat, Leningrad, 1951).

[2] H. Maske and H. Wolff, Acta Haematol. 95, 287 (1953).

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COMPLEX FORMATION BY BLOOD SERUM PROTEINS AS A RESULT OF THEIR REDUCTION BY CYSTEINE IN AN ACID MEDIUM

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According to present ideas [1], the antibody molecule differs from the molecule of normal globulin only in the structure of some portion of its surface which determines its specific properties and is called the antideterminant.

Since the disulfide structure determines to a considerable degree the stability of the structure of globular proteins [2], it seems interesting to consider how treatment of the antibody with reducing agents which partly destroy this structure will affect the specific activity of the antibody, or, in other words, how such a deformation of the molecule will be reflected in the specific activity of the antibody.

We have observed that the precipitating properties of immune serum are destroyed to an appreciable extent by cysteine only when it acts on the protein in an acid medium. In this connection it seems interesting to study how the chemical and physicochemical changes of serum proteins occur as a result of reduction in an acid medium.

The present work is devoted to the electrophoretic analysis of whole blood serum and its purified γ-globulin fraction treated in this way.

MATERIALS AND METHODS

In the experiments we used: normal beef serum and the albumin and γ-globulin fractions obtained from it by salting out with ammonium sulfate, horse antidiphtheria antitoxin and Flexner immune serum from donkeys.

As the reducing agent we used crystalline cysteine hydrochloride. The serum was treated with different concentrations of cysteine at a series of pH values, with a length of contact of serum with cysteine of one hour at room temperature, after which the serum was dialyzed in the cold against a large volume of veronal-medinal buffer, pH 8.6, and μ 0.1 for 35-40 hours.

The experiments were carried out in an apparatus for free electrophoresis made by Hilger with a Philpot-Swenson optical system. Observation of the optical boundaries was carried out in monochromatic green light. All the experiments were run in a buffer with the above composition at 4°. Electrophoresis was carried out at 12 ma and 5.75 v/cm.

Determination of the mobility of the protein fractions was carried out (with a descending boundary) by the formula:

$$U = \frac{l}{0{,}7 \cdot t \cdot E},$$

where l is the distance from ϵ to the given boundary in cm, 0.7 the enlargement of the apparatus, $\underline{t}$ the time in seconds, and E the potential gradient.

The relative concentration of the protein components in the electrophoretogram was determined by the method described by Tiselius and Kabat [3] and expressed in weight percent.

When we compared the electrophoretograms obtained in the different experiments, we used for analysis protein solutions of the same concentration (20 mg per ml). Photoexposure was carried out after the same time interval from the start of the electrophoresis and at the same position of inclination of the slit.

RESULTS

In Fig. 1 we give the electrophoretogram of antidiphtheria horse serum 90 minutes after beginning electrophoresis. There are four major components in this serum (excluding α-globulin), whose mobilities, calculated as in the other cases from the descending boundary in three parallel experiments, are shown in Table 1. It is important to remark that here, as in the remaining experiments, we do not get complete electrophoretic separation of all the serum proteins, for we are interested only in getting the greatest mobility of the components albumin and γ-globulin. When we compare the above electrophoretogram with the electrophoretogram of antidiphtheria horse serum treated with 1.66% cysteine at pH 3.7 (Fig. 2) we can see a very great difference. Actually, in this electrophoretogram we see a new D-component with a mobility close to that of γ_1-globulin, along with a considerable lessening of the peak for albumin (~33%) and β-globulin and complete disappearance of γ_1- and γ_2-globulins.

Fig. 1. Electrophoretogram of horse immune serum. Ascending boundary, 90 min electrophoresis.

This change was not found when we treated horse serum with 1.66% cysteine at pH 4.7.

Fig. 2. Electrophoretogram of horse immune serum treated with 1.66% cysteine at pH 3.7. Ascending boundary, 90 min electrophoresis.

As a result of the reduction by 1.66% cysteine at pH 3.7 (Figs. 3, 4) normal beef serum also shows the formation of a new D-component with a mobility intermediate between α- and β-globulins (Table 2). Simultaneously there is a decrease in the peak for albumin (~29%) and disappearance of α- and β-globulins. As to the peak of γ-globulin, this keeps its original mobility, but is greatly decreased in size (~50%). At the same time we see a considerable electrophoretic heterogeneity of the γ-globulin residue and its especially slow separation from the D-component (full separation does not occur even after three hours electrophoresis).

After reduction by 1.66% cysteine at pH 4.7, normal beef serum does not change its electrophoretic picture from that of untreated serum.

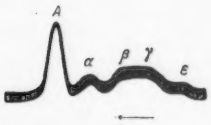

Fig. 3. Electrophoretogram of normal beef serum. Descending boundary, 70 min electrophoresis.

After electrophoresis of beef serum treated only with HCl to pH 3.7 (Fig. 5) there is no new electrophoretic component, no change in size of albumin peak, and only a "smearing" of the globulin portion of the electrophoretogram. As a result of treatment by 1.66% cysteine at pH 3.5, a mixture of beef γ-globulin and albumin with a small admixture of α-globulin, in the ratio 1:1 (Figs. 6, 7) showed changes analogous to those observed in whole serum, with full disappearance of γ-globulin and a new D-component composed of more than 79% of all the protein of the treated mixture. At the same time, treatment of isolated beef γ-globulin with 1.66% cysteine at pH 3.5 produced no change in its mobility or in the size of its peak compared to the control.

Reduction of immune donkey serum at pH 3.7 (Table 1) caused a decrease in its albumin peak along with a merging of all the globulin components of the serum into one very diffuse peak which did not separate even after 3.5 hours of electrophoresis.

When the changes which occurred in the electrophoretic picture of serum as a result of reduction were analyzed, they could be well enough explained as the result of complex formation by albumin and globulin. Actually, the appearance of a new serum component with mobility between γ-globulin and albumin is accompanied by full or almost full disappearance of γ-globulin and a greater or smaller decrease in the albumin peak. It must be emphasized that the action of cysteine under these conditions on isolated γ-globulin does not change its

TABLE 1

Electrophoretic Mobility and Relative Concentration of the Protein Components of Donkey and Horse Immune Serum Treated With Cysteine at Different pH

Type of serum	Electrophoretic mobility $x/-10^{-5} \cdot cm^2 \cdot sec^{-1} \cdot V^{-1}$						Relative concentration of protein components (wt %)					
	albumin	globulins				D	albumin	globulins				D
		α	β	γ_1	γ_2			α	β	γ_1	γ_2	
Unchanged horse serum	7.8	–	5.3	3.2	1.3	–	24.0	–	19.0	47.2	9.8	–
Horse serum treated with 1.66% cysteine at pH 3.7	7.6	–	5.4	–	–	3.4	16.0	–	9.2	–	–	74.8
Horse serum treated with 1.66% cysteine at pH 4.7	7.8	–	5.3	3.2	1.3	–	24.2	–	19.0	47.0	9.8	–
Unchanged donkey serum	8.9	6.3	5.3	–	2.9	–	25.2	21.7	7.2	–	45.9	–
Donkey serum treated with 1.66% cysteine at pH 3.7	8.8	–	–	–	–	3.9	19.8	–	–	–	–	80.0
Donkey serum treated only with HCl at pH 3.7	8.8	6.5	5.3	–	2.7	–	25.1	13.8	11.7	–	49.4	–

Fig. 4. Electrophoretogram of normal beef serum treated with 1.66% cysteine at pH 3.7. Descending boundary, 65 min electrophoresis.

Fig. 5. Electrophoretogram of normal beef serum treated only with HCl at pH 3.7. Descending boundary, 65 min electrophoresis.

Fig. 6. Electrophoretogram of a mixture of beef serum albumin and γ-globulin. Descending boundary, 65 min electrophoresis.

Fig. 7. Electrophoretogram of a mixture of beef serum albumin and γ-globulin treated with 1.66% cysteine at pH 3.5. Descending boundary, 65 min electrophoresis.

TABLE 2

Electrophoretic Mobility and Relative Concentration of Protein Components of Normal Beef Serum Reduced by Cysteine at Different pH

Type of serum	Electrophoretic mobility $x/-10^{-5} \cdot cm^2 \cdot sec^{-1} \cdot V^{-1}$					Relative concentration of protein components (in weight %)				
	albumin	globulins			D	albumin	globulins			D
		α	β	γ			α	β	γ	
Unchanged serum	9.5	7.0	4.4	3.1	–	36.0	15.0	20.0	29.0	–
Serum treated with 1.66% cysteine at pH 3.7	9.5	–	–	3.1	5.9	25.7	–	–	14.3	60.0
Serum treated with 0.83% cysteine at pH 4.8	9.5	7.0	4.4	3.1	–	34.8	14.8	21.5	28.9	–
Serum treated with HCl to pH 3.7	9.5	6.37	–	–	–	35.9	34.0	β + γ 30.1		–
System of albumin and γ-globulin	9.38	6.0	–	3.3	–	27.2	9.6	–		63.2
System of albumin and γ-globulin treated with 1.66% cysteine at pH 3.5	9.38	–	–	–	4.4	20.9	–	–	–	79.1
Serum treated with 1.66% cysteine and MIA	8.9	5.8	–	3.1	–	36.7	27.1	β + γ 36.2		–
Serum treated with 1.66% cysteine, dialyzed, and treated with MIA	8.9	–	–	3.1	5.0	33.7	–	–	20.3	46.0

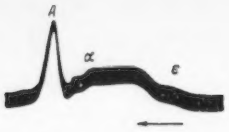

Fig. 8. Electrophoretogram of normal beef serum treated with 1.66% cysteine at pH 3.7 and MIA. Descending boundary, 65 min electrophoresis.

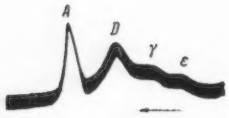

Fig. 9. Electrophoretogram of normal beef serum treated with 1.66% cysteine, dialyzed and submitted to the action of MIA. Descending boundary, 65 min electrophoresis.

electrophoretic properties, and the appearance of the new D-component is possible only in the presence of albumin. Complex formation of albumin and globulin is not the result of action of acids on the serum, as the corresponding control experiments showed.

What is the mechanism of complex formation of albumin and globulin? According to our ideas, complex formation of albumin and globulin occurs because when the protein is reduced, liberation of sulfhydryl groups by the reducing agent takes place. As to the nature of the bonds, probably albumin and globulin molecules combine in the complex chiefly by disulfide bonds formed as a result of reoxidation of the sulfhydryl group.

If the process actually proceeds in this way, then the process may be hindered by blocking the SH groups after reduction of the protein, but before the excess reducing agent is removed.

As such a blocking agent we chose monoiodoacetate (MIA) which irreversibly alkylates only the SH groups of proteins. KH_2COO^- + protein-SH → protein-S-CH_2COO^- + H^+ + I^+. Usually this reaction goes at pH 7-8, temperature from 0 to 25° in 0.5-2 hours.

The experiments were run as follows: serum reduced in the usual way was treated with an excess of 0.5 N neutral solution of MIA one hour after the introduction of the reducing agent, and the pH of the reaction mixture was carefully brought to 7.0. After a half-hour exposure at room temperature, the serum was dialyzed for a long time against a veronal-medinal buffer, pH 8.6 and μ 0.1, after which it was studied electrophoretically. Preliminary experiments showed that the concentrations of MIA used did not change the electrophoretic picture of untreated serum and decreased only slightly the mobility of the albumin fraction.

The resulting data (Fig. 8) fully confirmed our ideas. Actually, when the experiments were run thus, the albumin-globulin complex did not form and the only electrophoretic changes observed agreed fully with those produced on serum by the action of acid alone (Fig. 5), for we could not exclude the action of this factor.

In this connection it was interesting to determine whether MIA could act on the already formed albumin-globulin complex. If albumin and globulin are actually combined by disulfide bonds, then MIA, a reagent for the SH group, should not destroy the already formed complex.

To test this assumption, portions of beef serum were treated in the usual way with 1.66% cysteine at pH 3.7 and then dialyzed against a veronal buffer at pH 8.0. After dialysis the serum was divided into two parts, one of which was treated with MIA which was added in excess. After 90 minutes exposure at room temperature the treated and untreated portions of serum were dialyzed separately against a veronal-medinal buffer, pH 8.6 and μ 0.1 in the cold for 30 hours and then submitted to electrophoresis.

The electrophoretograms of the control test did not differ from those obtained earlier: in them we observed the albumin-globulin complex along with the corresponding decrease in the peak of albumin and γ-globulin. As to the electrophoretograms of the reduced serum treated with MIA (Fig. 9), we could see from analysis of it a partial splitting of the albumin-globulin complex accompanied by a rise in the albumin peak which was now only 6% less than normal, and a notable increase in the γ-globulin peak (42%). Analogous results were also obtained in experiments with donkey immune serum.

As serological study showed, the immune serum lost its precipitating properties as a result of reduction and after treatment with MIA again regained the ability to precipitate specific antigens.

Thus, the data from these experiments shows that the new component formed in reduction of serum is an albumin-globulin complex for when it is destroyed by the action of MIA there is an increase in both albumin and globulin components of the serum.

On the other hand, these experiments cast doubt on the possible union of the albumin and globulin components in the complex through a disulfide bond, and the question of the nature of the bond between albumin and globulin remains open.

DISCUSSION OF RESULTS

As the experiments with MIA showed, complex formation of albumin and globulin occurs chiefly because of the sulfhydryl group which is formed by reduction of the protein by cysteine. However, such a process occurs only when the protein is reduced in acid medium and does not occur when the latter is treated with cysteine at a pH near neutral. Evidently, the conditions of the acid medium lead to deformation and swelling of the globular protein [2] which makes it easy for the cysteine to attack the disulfide bond of the protein. This idea is confirmed by the work of Marcus and Karush [4] who showed especially that the swelling of globular proteins under the influence of detergents (decylsulfate) permitted the rupture of a much larger number of disulfide links in the reduction of proteins than when the reducing agent acted alone.

It is important to say that complex formation of albumin and globulin has been observed electrophoretically under the influence of a number of different agents on serum, for example: heating to 65° [5], action of ultraviolet rays [6], photooxidation [7], and some others. In all these cases along with the electrophoretic and immunological experiments, there has been observed a deep disturbance in precipitating power of the antiserum as a result of such action.

The above data along with our results indicate an important parallelism and permit us to join with those authors [7, 8] who believe that complex formation of immune proteins with albumin results in deep disturbance of the serological properties of the antisera.

SUMMARY

The action of a reducing agent (cysteine) on serum in an acid medium leads to considerable changes in the physicochemical properties of the protein which accompany complex formation by albumin and globulin. The albumin-globulin complex occurs because of chemical bonds, in the formation of which an important role is played by the liberation of sulfhydryl groups in the proteins by the action of the reducing agent.

LITERATURE CITED

[1] L. Pauling, J. Amer. Chem. Soc. 62, 2643 (1940).

[2] L.B. Gorbacheva, S.E. Bresler and S.Ya. Frenkel, Biokhimiya 22, 70 (1957).*

[3] A. Tiselius and E.A. Kabat, J. Exptl. Med. 69, 119 (1939).

[4] G. Marcus and F. Karush, J. Amer. Chem. Soc. 79, 134 (1957).

[5] J. Van der Scheer, R.W.G. Wyckoff and F.L. Clarke, J. Immunol. 40, 39 (1941).

[6] B.D. Davis, A. Hollaender and I.P. Greenstein, J. Biol. Chem. 146, 663 (1942).

[7] A. Tyler and S.M. Swingle, J. Immunol. 51, 339 (1945).

[8] A. Kleczkowski, Brit. J. Exptl. Pathol. 22, 188 (1941); 35, 402 (1954).

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*Original Russian pagination. See C.B. translation.

THE PATHS OF DEAMINATION OF NATURAL AMINO ACIDS IN LIVER AND KIDNEY TISSUES OF BIRDS

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We have shown earlier [1] that in surviving tissues of bird liver and kidney the deamination of many L-amino acids (including aromatic and diamino acids) and glycine goes more intensively than in mammalian tissues.

The amount of deamination of different amino acids differs sharply, depending on structure and also on the organ and type and individual characteristics of the bird. These results permit us to assume that in bird tissues deamination of different amino acids proceeds by different enzyme systems.

The ideas of Braunshtein on the preferential role of "transdeamination" with participation of transaminases and glutamic dehydrogenase in the formation of ammonia from L-amino acids in the tissues of mammals have obtained experimental confirmation [2-9].

At the present time we can consider it established that the deamination of L-alanine, L-aspartic acid, and a number of other natural amino acids in mammalian tissue proceeds by transdeamination [10, 11].

The mechanism of deamination of natural amino acids in bird tissue is almost unstudied. In this connection it seems interesting to determine how much the transamination reaction plays a part in these processes in birds. The results are given below.

EXPERIMENTAL

For the experiments we used liver and kidney tissues of pigeons, chicks and turkeys, young and mature. The experiments were carried out on sections and homogenates of liver and kidney and with extracts of acetone-treated liver. (The detailed conditions of carrying out the experiments were described earlier [1].) The purity of the amino acid preparations used was tested chromatographically; the preparations contained no free ammonia. As the inhibitor we used hydroxylamine hydrochloride (commercial preparation), potassium fluoroacetate obtained in the laboratory from methylfluoroacetate, mesotartaric and tartaric acids.*

To explain the role of the transamination reaction in the deamination of L-amino acids we used poisons which could suppress the transamination reaction. One of these poisons, hydroxylamine, as is known, binds the CO groups of pyridoxal, that is, the activating groups of transaminase. In the work of Braunshtein and co-workers [12] it was shown that hydroxylamine causes a competitive inhibition of transamination. Thus, hydroxylamine in experiments with purified glutamic-alanine transaminase and its substrates gave 35% inhibition of the transamination reaction if the ratio of concentration of hydroxylamine and α-ketoacid was 1:10 and 80-90% at a ratio of 1:5.

Another inhibitor used in the work, fluoroacetic acid, is changed in the tissues to fluorocitric acid. The latter inhibits the action of aconitase and this blocks the transformation of citric acid to ketoglutaric acid in the

*The preparations of mesotartaric and tartaric acids were obtained from R.M. Azarkh of the Institute of Biological and Medical Chemistry, Academy of Medical Sciences, USSR.

TABLE 1

Effect of Hydroxylamine and Fluoroacetate on Deamination of the L-Isomer of Analine, Aspartic and Glutamic Acids in Bird Tissues

Type of bird	Tissue preparation	ΔNH_3 in μmoles/g		% Inhibition	ΔNH_3 in μmoles/g		% Inhibition
		control	hydroxylamine		control	hydroxylamine	
		L-Alanine					
Pigeon	Kidney section	70.2	3.2	95	64.3	47.5	26
"	The same	63.0	0	100	91.3	42.2	54
"	Liver section	19.2	0	100	34.0	13.0	62
"	The same	22.9	0	100	15.1	0	100
"	Liver homogenate	30.9	12.4	60	30.9	0.8	97
Chick	Liver section	11.1	0	100	9.6	0	100
"	The same	43.4	6.7	85	–	–	–
Turkey poult	Kidney section	122.8	29.1	76	122.8	83.1	24
" "	Liver section	23.7	18.6	22	–	–	–
		L-Aspartic acid					
Chick	Kidney section	136.8	45.8	67	136.8	83.1	46
"	The same	59.4	0	100	59.4	32.9	45
"	Liver homogenate	16.6	1.6	90	–	–	–
		L-Glutamic acid					
Pigeon	Kidney section	39.9	39.0	3	31.5	47.6	(activated)
"	The same	47.3	54.0	(activated)	40.5	44.8	"
	The same	–	–	–	25.9	44.8	"
Turkey poult	Kidney section	32.6	52.5	(activated)	–	–	–

tricarboxylic acid cycle. In the presence of fluoroacetate the transformation of L-amino acids added to the tissues into glutamic acid by the transamination reaction is suppressed as a result of inhibition of formation of ketoglutaric acid in the process of cell respiration [10, 11, 13, 14]. In our experiments hydroxylamine was added to incubating samples with a final concentration of $4 \cdot 10^{-3}$ M, potassium fluoroacetate in a concentration of $5 \cdot 10^{-3}$ M.

The results of the effect of hydroxylamine and fluoroacetate on the amount of deamination of L-alanine, L-aspartic acid and L-glutamic acid in bird tissues are given in Table 1. These results indicate the sharp inhibition of deamination of L-alanine and L-aspartic acid under the influence of both hydroxylamine and fluoroacetate in sections and homogenates of kidney and liver tissues of birds. The inhibition of deamination of L-alanine and L-aspartic acid in the presence of hydroxylamine in a number of experiments reached 100%, and in only one experiment with turkey liver section was the inhibition of deamination of L-alanine especially low (22%). The inhibition of deamination of these amino acids by fluoroacetate was somewhat less complete than when hydroxylamine was used: on the average it was 66% for L-alanine and about 45% for L-aspartic acid.

In the tests with glutamic acid, as was expected, in no case was there inhibition of deamination by hydroxylamine or fluoroacetate. It is worthy of note that the increase in ammonia nitrogen in the tests incubated in the presence of hydroxylamine or fluoroacetate is even higher than in the control samples. In the experiments with hydroxylamine this effect is evidently due to the fact that the equilibrium of the reverse reaction, catalyzed by glutamic dehydrogenase, is shifted toward more active deamination of glutamic acid as a result of binding α-ketoglutaric acid as its oxime. An analogous result is produced by increasing the rate of oxidation of α-ketoglutaric acid in the samples which contain fluoroacetate, in which the tricarboxylic acid cycle is blocked at the aconitase level which blocks the oxidation of citric acid and its precursors in the cycle as substrates of cell respiration [13].

The data of Table 1 show that in surviving bird liver and kidney tissues the deamination of L-glutamic acid occurs directly under the influence of glutamic dehydrogenase, while the deamination of L-alanine and L-aspartic acid occurs as the result of transamination, as in mammalian tissue [2, 3, 5, 6, 9-11, 14].

In experiments with B_6 avitaminosis in chicks we obtained supplementary indications of the role of transamination in the deamination of L-alanine in bird tissues.* In this series of experiments we compared the values for deamination of L-alanine and L-glutamic acid in kidney sections of control and B_6-deficient chicks.

The experiments were carried out on 4-9-day-old chicks, weight 30-60 g, maintained on a synthetic diet described by Oleson et al. [15] with some modifications. To the diet were added all the vitamins except pyridoxine. The control chicks received 75 g of pyridoxine per chick daily. Development of the avitaminosis was determined by external signs (loss of weight, general condition of the chicks) and was verified by destruction of transamination processes between L-glutamic acid and pyruvic acid in liver homogenates. In 7-8 days after placing the chick on the synthetic diet, when the B_6 avitaminosis had reached a high level (fall in activity of glutamic-alanine transaminase to 60-75%), we carried out experiments on deamination of L-alanine and L-glutamic acid in kidney sections of control and vitamin-deficient chicks; carrying out experiments with liver slices was not expedient because of their slight permeability to glutamic acid.

As the data in Table 2 show, the amount of deamination of L-alanine in kidney slices in the control chicks varied from 43-108 μmoles per g of tissue, and in the B_6-deficient chicks from 16 to 77 μmoles/g. The average value of deamination of L-alanine in control chicks was 71.5 μmoles/g, and in the B_6-deficient chicks was 45 μmoles/g, that is 35% less. The value for deamination of L-glutamic acid in the control and deficient chicks was almost the same: in the control group it averaged 30 μmoles/g, in the B_6-deficient group, 27 μmoles/g. The results of these experiments agree well with the results obtained by Sinitsyna [9] in a study of the effect of insufficient vitamin B_6 on the deamination of L-alanine and L-glutamic acid in rat tissues.

TABLE 2

Deamination of L-Alanine and L-Glutamic Acid in Kidney Slices of Control and B_6-Deficient Chicks

Control chicks				B_6-Deficient chicks			
Expt. No.	No. of days on diet	L-alanine, ΔNH_3 in μmoles/g	L-glutamic acid, ΔNH_3 in μmoles/g	Expt. No.	No. of days on diet	L-alanine, ΔNH_3 in μmoles/g	L-glutamic acid, ΔNH_3 in μmoles/g
1	8	72	39	1	8	16	32
2	13	64	15	2	11	71	27
3	14	56	11	3	12	37	55
4	11	73	31	4	14	47	34
5	4	108	42	5	11	25	19
6	8	62	46	6	11	47	32
7	8	69	10	7	11	24	41
8	5	57	57	8	9	68	23
9	3	43	30	9	8	55	10
10	4	92	24	10	11	77	9.6
11	5	86	28	11	12	49	31
12	10	66	26	12	13	32	14
13	12	86	35	–	–	–	–
14	13	71	30	–	–	–	–
Average:		71.5	29.5	Average:		45	27

We studied further the deamination of L-alanine and L-aspartic acid in the presence of mesotartaric acid. According to the data of Quastel and Scholefield [16], mesotartaric acid blocks the formation of oxaloacetic acid from pyruvic acid and CO_2 and thus inhibits the transformation of pyruvate into citric acid in the tricarboxylic

*The experiments with B_6 avitaminosis in chicks were carried out in our laboratory by Student of the Biological Faculty, Moscow State University K.Yu. Khorunzhaya.

acid cycle; the natural form of tartaric acid (the D-isomer) has no inhibiting action on the carboxylation of pyruvic acid. Hence we expect that mesotartaric acid should inhibit the process of transdeamination of D-alanine while L-tartaric acid should show no such action. As the results of the experiment show (Table 3) mesotartaric acid in concentration of 10^{-2} M causes considerable inhibition of the deamination of L-alanine in bird liver and kidney tissues. The degree of inhibition from experiment to experiment varies, being 40-50% for chick and pigeon kidney slices and 40-100% for liver homogenates of chicks.

TABLE 3

Effect of Mesotartaric Acid on Deamination of L-Alanine and L-Aspartic Acid in Bird Tissues

Type of bird	Tissue preparation	ΔNH_3 in μmoles/g		% Inhibition
		control	mesotartaric acid	
	L-Alanine			
Pigeon	Kidney slice	39.9	19.4	51
Chick	" "	56.7	34.4	39
"	Liver homogenate	50.5	3.6	93
"	The same	35.5	0	100
"	The same	33.3	20.2	40
	L-Aspartic acid			
Pigeon	Kidney slice	56.9	45.2	21
Chick	" "	79.1	57.6	27
"	Liver homogenate	16.6	22.4	—

Considering the data of Quostel on the mechanism of inhibition by mesotartaric acid [16] we can expect that mesotartaric acid will not destroy the transdeamination of L-aspartic acid, since the oxaloacetic acid formed from it can pass continuously into the tricarboxylic acid cycle in the presence of mesotartaric acid, so that formation of ketoglutaric acid is not prevented. As Table 3 shows, deamination of L-aspartic acid when mesotartaric acid is added is either not destroyed or is inhibited only slightly, by 25% (in kidney slices). This slight inhibition depends, perhaps, on partial inhibition of enzymatic decarboxylation of oxaloacetic acid to pyruvic due to which, in some degree, the formation of ketoglutaric acid in the respiratory cycle becomes difficult.

D-Tartaric acid, as distinguished from mesotartaric acid, as a rule does not destroy the transdeamination of L-alanine; deamination of L-glutamic acid, as would be expected, is not inhibited either by D-tartaric or mesotartaric acids (Table 4).

TABLE 4

Effect of Meso- and D-Tartaric Acids (10^{-2} M) on Deamination of L-Alanine and L-Glutamic Acids by Chick Liver Slices

Amino acid	ΔNH_3 in μmoles/g		
	control	mesotartaric acid	D-tartaric acid
L-Alanine	15.1	10.6	15.4
L-Glutamic acid	11.2	22.1	24.0

The intensity of deamination of L-glutamic acid in the presence of mesotartaric or D-tartaric acids not only is not inhibited, but is even increased considerably. The reason for this effect is probably the same as for the action of fluoroacetic acid. Thus, the data obtained in the experiments with mesotartaric and D-tartaric acids also confirms that L-alanine and L-aspartic acids are deaminated in bird tissues by transdeamination.

TABLE 5

Effect of Hydroxylamine and Fluoroacetate on Deamination of Phenylalanine and Leucine in Bird Tissues

Tissue preparation	Control	Hydroxylamine		Fluoroacetate	
	ΔNH_3, in μmoles/g	ΔNH_3, in μmoles/g	% inhibition	ΔNH_3, in μmoles/g	% inhibition
L-Phenylalanine					
Pigeon liver slice	37.3	35.9	4	0	100
Turkey liver slice	45.6	0	100	27.8	39
Chick kidney slice	47.7	5.1	94	40.9	14
The same	7.8	0	100	–	–
Pigeon liver homogenate	11.7	18.3	0	13.6	0
Extract of acetone-treated turkey liver	5.8	4.7	19	–	–
D-Phenylalanine					
Pigeon kidney slice	218.0	216.0	1	–	–
The same	177.7	–	–	186.3	0
Turkey liver slice	27.5	26.4	4	–	–
Pigeon liver slice	169.0	–	–	173.4	0
L-Leucine					
Turkey liver slice	38.2	33.1	13	49.7	0
Chick kidney slice	11.6	8.6	26	8.1	30
Turkey liver homogenate	13.1	17.6	0	24.0	100
The same	16.2	0	100	0	100
The same	5.1	6.1	0	0	100
Chick kidney homogenate	10.9	3.8	65	0	100
The same	25.9	0.6	98	33.6	0
Pigeon liver homogenate	1.9	0	100	0	100

We also studied the effect of hydroxylamine and fluoroacetate on deamination of other L-amino acids in bird tissues. The results of the experiments on the action of these poisons on the deamination of L-phenylalanine and L-leucine are given in Table 5.

The results of these experiments were not simple. Both hydroxylamine and fluoroacetate in about half the experiments inhibited the oxidation of L-leucine fully or more than 60%, and in the other half of the experiments did not inhibit it at all, or only slightly (less than 40%), and the same relative results were obtained in the experiments on deamination of L-phenylalanine. Often in the same experiment one of the two inhibitors studied fully suppressed the deamination process and the other showed no action or caused only a slight inhibition of deamination of the same amino acid. It should be said that in the experiments with liver homogenate, and also with extracts of acetone-treated liver, neither hydroxylamine nor fluoroacetate, as a rule, inhibited deamination of L-phenylalanine. The observed differences in effect of inhibitors of transamination on formation of ammonia from L-phenylalanine and L-leucine in bird tissues cannot be due to chance variations in the conduct of the experiments, since in the perfectly analogous experiments with L-alanine and L-aspartic acid the same poisons regularly showed an inhibiting action, and in the study of their effect on the deamination of simple natural amino acids (glycine, L-isomers of valine, isoleucine, cyclic amino acids and diamino acids) the absence of inhibition was established for all. In the same way, hydroxylamine and fluoroacetate under these experimental conditions never inhibited direct deamination of L-phenylalanine (Table 5) and other L-amino acids in bird liver and kidney slices.

These data permit us to assume that in bird liver and kidney tissues the deamination of L-phenylalanine and L-leucine can occur in two ways, directly and indirectly, and the predominating role is taken, apparently, by the indirect mechanism, that is, transdeamination. These two mechanisms, depending on composition of the tissue, and characteristics of the individual bird, occur to a greater or lesser extent. With respect to L-phenylalanine, the fact that this amino acid is relatively actively deaminated in extracts of acetone-treated livers of

turkeys and this deamination is not inhibited by the transdeamination inhibitors shows the presence in the bird tissues of a direct process for oxidative deamination of this amino acid [1, 17, 18]. Hydroxylamine and fluoroacetate have no effect on the deamination of other natural amino acids in bird liver and kidney.

In previous work [1] it was shown that glycine is deaminated only in turkey liver slices. It was interesting to determine the relation of the process of deamination of glycine to benzoic acid, which, as is known, in concentrations of 0.02 M gives 100% inhibition of the action of purified glycine oxidase isolated from rat livers by Green et al. [22]. In our experiments in the presence of sodium benzoate (final concentration in the sample $2 \cdot 10^{-2}$ M) we never found complete suppression of glycine deamination in turkey liver slices (which contained from 11 to 19 μmoles/g); inhibition in only one experiment reached 77% and in the others varied from 19 to 50%.

We showed earlier [1] that in turkey liver tissues there is intense deamination of diamino acids, described by Boulanger and Osteux [17], and also of L-tryptophan, and this amino acid is deaminated fast enough not only in surviving tissues, but also in extracts of acetone-treated liver, the deaminating activity of which is almost unchanged even after long keeping of the acetone-treated preparation.

In a series of experiments we studied the action of hydroxylamine and fluoroacetate and also sodium benzoate on deamination of L-lysine and L-tryptophan in extracts of turkey liver. We showed that none of these agents had any practical effect on the rate of formation of ammonia from these amino acids.

Identification of the deamination products of L-arginine and L-tryptophan in extracts of turkey liver. The nature of the soluble deaminating enzymes in bird liver extracts has not been sufficiently studied. The oxidative mechanism of deamination of L-arginine and L-tryptophan in such extracts was confirmed by us by identification of the α-ketoacids which were formed. Recently, in analogous experiments Boulanger and Osteux [18] identified the products of oxidative deamination of L-lysine and L-arginine by a chromatographic method.

Meister [19] earlier described the formation of α-keto-δ-guanidinovaleric acid ("α-ketoarginine") by deamination of L-arginine with the L-amino acid oxidase from snake venom. We used his method for the preparative isolation of α-ketoarginine in experiments with extracts of acetone-treated turkey liver.

25 g of acetone-dried powder from turkey livers was extracted with 100 ml of Na pyrophosphate buffer (pH 8.2, M/30). The resulting extract in which active catalase was present, was incubated for seven hours with shaking in an O_2 atmosphere with 1.12 g of L-arginine monohydrochloride; determination of free ammonia showed that 1.08 g of arginine·HCl had undergone deamination. The incubation mixture was dialyzed against several changes of distilled water; the dialysate was concentrated in a vacuum and the ketoacid was precipitated from it with absolute alcohol in the cold. The dried precipitate after recrystallization from hot water weighed 0.42 g (58% of the calculated amount). The resulting product was identified as α-keto-δ-guanidinovaleric acid by the method of paper chromatography [19] by the following evidence: positive Sakaguchi reaction and negative ninhydrin. With a hydrochloric acid solution of 2,4-dinitrophenylhydrazine this compound gives a hydrazine hydrochloride in the form of yellow, silky needles which when heated change with decomposition to an orange substance at 210° (according to the literature it decomposes at 216°); on further heating it does not change.

We further used the resulting α-ketoarginine in amination experiments in pigeon liver homogenates and showed the formation from it of arginine.

As already reported [1], when L-tryptophan is deaminated by bird liver preparations we find the appearance in the incubation mixture of a mixture of yellow-red pigments whose intensity parallels the amount of ammonia formed [20]. It was natural to assume that the color depends on products of a secondary transformation of β-indolylpyruvic acid which formed in the oxidative deamination of tryptophan. Indolylpyruvic acid in water solution in air at room temperature is rapidly decomposed with formation of indole acetic acid and other oxidation products; some of them are colored. Therefore, there is no possibility of isolating indolylpyruvic acid preparatively from the incubation sample after deamination of L-tryptophan by turkey liver extracts. For identification of the products of tryptophan deamination we used the method of Bentley et al. [21] who studied the chemical and physiological properties of indolylpyruvic acid.

The filtrates of incubated and unincubated samples which contained tryptophan and extracts of acetone-treated liver, after precipitation of proteins by metaphosphoric acid were submitted to single-phase paper chromatrography on Whatman No. 1 paper; the solvent (1 part glacial acetic acid + 3 parts distilled water) was passed for 3-4 hours. As the indicator on the chromatogram we used a solution of indole acetic acid. Parallel chromatograms were developed with one of the following reagents:

1. Ammoniacal $AgNO_3$ [50 ml of NH_3 solution (d = 0.88) in 100 ml of a 1 N solution of $AgNO_3$].

2. Erlich reagent [2 g dimethylaminobenzaldehyde dissolved in a mixture of 20 ml of hydrochloric acid (d = 1.18) and 80 ml of alcohol].

3. "Nitrous solution" [1 g KNO_2 and 20 ml HNO_3 (d = 1.42) dissolved in water to 200 ml].

4. Salkowski reagent (2 ml of 0.05 N solution of $FeCl_3$ + 180 ml of 5% $HClO_4$ solution).

On the chromatogram indolylpyruvic acid is rapidly colored black by reagent 1 due to reduction of the silver: indole acetic acid slowly reduces silver (after 12 hours). The other reagents color the spots of indolylpyruvic acid different colors, which do not agree with the colors of the spots of indole acetic acid and the other oxidation products [21].

In our experiments with chromatography we usually developed two spots, identified as indolylpyruvic acid (R_f = 0.38) and indole acetic acid (R_f 0.71); the other intermediate oxidation products of indolylpyruvic acid described by Bentley were not seen on our chromatogram. The results of these experiments allow us to conclude that in the deamination of L-tryptophan by the enzymes of the turkey liver extracts indolylpyruvic acid is formed and is quickly oxidized to indole acetic acid; the characteristic color of the incubated samples depends on products of the decomposition of these acids which do not give clear spots on a chromatogram.

DISCUSSION OF RESULTS

These results permit us to conclude that in bird livers and kidneys deamination of natural amino acids occurs by different enzyme systems. L-Glutamic acid in bird liver and kidney tissues undergoes direct oxidative deamination under the action of glutamic acid dehydrogenase. The deamination of L-aspartic acid and L-alanine occurs, as in mammalian tissue, as a result of transdeamination with participation of transaminase and glutamic dehydrogenase.

The mechanism of deamination of L-phenylalanine and L-leucine in bird tissues is not completely clear. Evidently for these amino acids parallel deamination mechanisms are possible, direct and indirect, by transamination. Deamination of the other L-amino acids and glycine in bird liver and kidney enzyme preparations, according to our data, is not suppressed by inhibitors which destroy the process of transdeamination (hydroxylamine, fluoroacetate). These amino acids are evidently deaminated in bird tissues by L-amino acid oxidase which is practically absent in mammalian tissue. Boulanger and Osteux [18] obtained from turkey livers a purified preparation of L-diamino acid oxidase and showed that it was active chiefly in respect of L-isomers of diamino acids and in less degree toward L-phenylalanine and L-tyrosine. In confirmation of the data obtained by these authors we have established that oxidative deamination of L-isomers (histidine and arginine) in bird liver and kidneys occurs directly. As the product of deamination of L-arginine we have prepared and isolated α-keto-δ-guanidinovaleric acid and for the first time have found active deamination of L-tryptophan in turkey liver tissue kept as an acetone-dried preparation [1]. The product of deamination of L-tryptophan was identified as indolylpyruvic acid which was quickly transformed into indole acetic acid. The question of the possibility of identifying the enzymes which deaminate the L-diamino acids and L-tryptophan still remains open.

In turkey livers we have observed an enzyme system which deaminates glycine (the same, but weakly active system occurs in turkey kidney and pigeon tissues). Deamination of glycine is found only in slices of these tissues; it is not destroyed in the presence of hydroxylamine and fluoroacetate and is only partly suppressed by sodium benzoate. Thus, this enzyme system differs somewhat in its properties from that described by Green and co-workers [22] as a glycine oxidase from rat kidney. The latter was weakly active under experimental conditions analogous to ours, but retained its activity in cell-free extracts and was more sensitive to benzoic acid.

SUMMARY

We have studied the effect of inhibitors which destroy the process of transdeamination, hydroxylamine ($4 \cdot 10^{-3}$ M), fluoroacetate ($5 \cdot 10^{-3}$ M), and mesotartaric acid (10^{-2} M), and also sodium benzoate (10^{-2} M), on the deamination of L-amino acids and glycine in bird liver and kidney tissues. The experimental data reported in the text lead to the following conclusions.

L-Glutamic acid in bird tissues undergoes direct deamination by the action of L-glutamic dehydrogenase. In the tissues the rate of this process is increased in the presence of hydroxylamine, fluoroacetate, or mesotartaric acid and is not changed in B_6 avitaminosis.

Deamination of L-alanine and L-aspartic acid in bird liver and kidney goes by transdeamination and is sharply inhibited in the presence of inhibitors which destroy this process and also in B_6 avitaminosis (deamination of L-alanine).

Deamination of L-phenylalanine and L-leucine in bird tissues can evidently occur directly or indirectly, and its suppression by the inhibitors of transdeamination varies in wide limits (from 0 to 100%).

Deamination of the remaining L-amino acids and glycine in bird tissues is not sensitive to transdeamination inhibitors and evidently occurs by direct action under the influence of amino acid oxidase which is absent or very weakly active in mammalian tissue.

Active deamination of glycine in turkey liver (and more weakly in turkey kidney and pigeon tissues) is found only when the cell structure is preserved (in slices); it is partly inhibited by sodium benzoate.

Extracts of acetone-treated turkey liver powder keep a high deaminating activity toward D-amino acids, L-diamino acids, and L-tryptophan, and to a lesser degree toward L-phenylalanine. Deamination of L-amino acids in turkey liver extracts is not inhibited by sodium benzoate. The oxidative mechanism of this process was confirmed by preparative isolation of α-ketoacids formed in the deamination of L-arginine and chromatographic demonstration of the formation of indolylpyruvic acid and indole acetic acid from L-tryptophan.

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LITERATURE CITED

[1] E.F. Efimochkina, Biokhimiya 23, 683 (1958).*

[2] A.E. Braunshtein and S.M. Bychkov, Biokhimiya 5, 261 (1940).

[3] A.E. Braunshtein and R.M. Azarkh, Biokhimiya 9, 337 (1944).

[4] A.E. Braunshtein, E.V. Goryachenkova and T.S. Paskhina, Biokhimiya 14, 163 (1949).

[5] J.L. Still, M.V. Buell and B.E. Green, Arch. Biochem. 26, 413 (1950).

[6] H. Nakada and S. Weinhouse, J. Biol. Chem. 187, 663 (1950).

[7] W. Knox and M. Le May-Knox, Biochem. J. 49, 686 (1951).

[8] T.T. Berezov, Doklady Akad. Nauk SSSR 86, 605 (1952).

[9] A.L. Sinitsyna, Biokhimiya 19, 80 (1954).

[10] R.M. Azarkh, VIII All-Union Congress of Physiology, Biochemistry, Pharmacology [in Russian] (Izd. AN SSSR, Moscow, 1955) p. 11.

[11] A.E. Braunshtein, VIII All-Union Congress of Physiology, Biochemistry, Pharmacology [in Russian] (Izd. AN SSSR, Moscow, 1955) p. 83.

[12] A.E. Braunshtein, R.M. Azarkh and Z.G. Mogilevskaya, Ukrain. Biokhim. Zhur. 27, 259 (1955).

[13] A.E. Braunshtein and R.M. Azarkh, Vopr. Med. Khim. 3, 380 (1957).

[14] A.E. Braunshtein, Chief Paths of Assimilation and Dissimilation of Nitrogen in Animals [in Russian] (Izd. AN SSSR, Moscow, 1957).

[15] J.J. Oleson, B.L. Hutchings and N.H. Sloane, J. Biol. Chem. 165, 371 (1946).

[16] J.H. Quostel and P.G. Scholefield, J. Biol. Chem. 214, 245 (1955).

[17] P. Boulanger and R. Osteux, Compt. rend. Acad. Sci. 241, 125 (1955).

[18] P. Boulanger and R. Osteux, Compt. rend. Acad. Sci. 241, 613 (1955).

[19] A. Meister, J. Biol. Chem. 197, 309 (1952); 206, 577 (1954).

*See English translation.

[20] T.L. Sourkes and E. Townsend, Canad. J. Biochem. and Physiol. 33, 735 (1955).

[21] J.A. Bentley, K.K. Farrar, S. Housley et al., Biochem. J. 64, 44 (1956).

[22] S. Ratner, V. Nocito and D.E. Green, J. Biol. Chem. 152, 119 (1944).

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THE SORPTION OF PROTEINS BY ION EXCHANGE RESINS

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The study of sorption of insulin by sulforesins [1] showed that with sufficient porosity, strongly swelling resins of the SBS type can adsorb a considerable amount of insulin but do not have a great sorption capacity for proteins with greater molecular weight such as blood serum proteins. On this principle we worked out a method for separating proteins by molecular weight with the help of a so-called molecular or ionic sieve. In order to determine the sorption of proteins with large molecular weights on sulforesins, we decided to use strongly swelling resins in very finely ground form, that is, to adsorb the proteins with use of a large sorbent surface.

For the preparation of such types of sorbent, the sulforesin SBS-3 with a swelling coefficient of six was ground for several hours in a ball mill. After sifting out the large particles, the main mass of powdered sorbent was fractionated by sedimentation. We collected the fraction of the resin which settled in water at a height of 40 cm during 15-30 minutes. For the study we used the squeezed out moist ionite, since otherwise, if it was dried it formed lumps which were difficult to disperse in solution. To calculate the sorption capacity for proteins we determined the moisture of the ionite dried at 80°. Since the solution passes very poorly through a column of such finely divided sorbent, all the experiments were carried out under static conditions. A definite weight of resin was placed in a solution contained in a vessel with a stirrer. Sorption took place with energetic mixing of the solution. To determine the residual concentration of the solution after sorption we separated the sorbent by centrifuging.

As Fig. 1 shows, sorption equilibrium was established in ten minutes on resin SBS-3 in the hydrogen form when adsorbing blood serum proteins from 0.001 M phosphate buffer solution, pH 7.4. The sorption capacity for the protein reached a considerable size, on the order of 100 mg/g of dry resin. For comparison, in Fig. 2 we give the curve for sorption of γ-globulin on a strongly swelling resin in the hydrogen form at a low rate of passing the solution (2 ml/cm^2 hour). The sorption equilibrium, as we see, is not established in one-hundred hours.

A study of the sorption of the simplest dipolar ions which we carried out earlier [2] showed the mechanism of the sorption of such ions by cationites. Sulfocationites in the hydrogen form adsorbed dipolar ions without exchange with an equivalent number of hydrogen ions according to the equation:

$$R_1SO_3^-H^+ + {}^+H_3NR_2COO^- = R_1SO_3^-{}^+H_3NR_2COOH, \quad (1)$$

where $R_1SO_3^-H^+$ is the sulfocationite in the hydrogen form, and ${}^+H_3NR_2COO^-$ is the dipolar ion (amino acid, peptide, protein). Corresponding to this mechanism the dipolar ion is changed to a cation which reacts with the negatively charged sulfo group of the sorbent. Thus, the electrostatic repulsion which had existed in this case disappears when the dipolar ion unites with the negative (or positive) charge of the sorbent. When the simplest dipolar ions are adsorbed on the salt form of the sulforesin, the dipolar ion does not change to a cation and so adsorption of an amino acid occurs at lower capacity compared to its sorption on the sulforesin in the hydrogen form [2].

The sorption of the majority of proteins on sulforesins follows the same rules (Table 1). Blood serum proteins are not adsorbed at all on the sulforesin SBS-3 in the sodium form but are adsorbed by the same resin in the hydrogen form. For some proteins this behavior, typical for dipolar ions, is not found. The sorption capacity for a number of proteins of the sulforesin in the sodium form approaches the sorption by the same resin in the

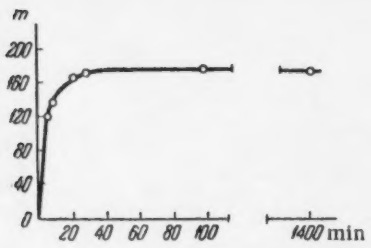

Fig. 1. Sorption of γ-globulin by finely ground resin SBS-3 in the hydrogen form under static conditions. m – Amount of adsorbed γ-globulin in mg/g.

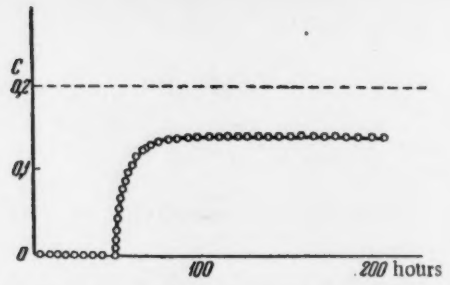

Fig. 2. Sorption of γ-globulin by SBS-3 resin (particle diameter 0.1-0.2 mm) under dynamic conditions at rate of flow of solution 2 ml/cm² hour. c – Concentration of γ-globulin in mg/ml. Concentration of starting solution 0.2 mg/ml.

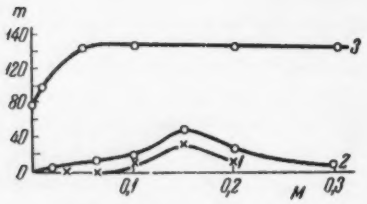

Fig. 3. Relation of sorption capacity for proteins of SBS-3 resin in the sodium form and concentration of Na phosphate buffer solution. m – Amount of adsorbed protein in mg/g; M – molarity of buffer solution; 1) serum albumin; 2) γ-globulin; 3) insulin.

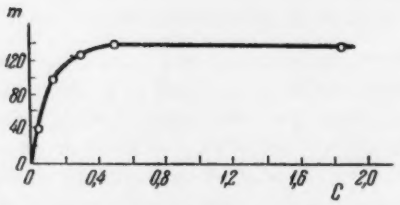

Fig. 4. Adsorption isotherm of serum albumin by resin SBS-3 in the hydrogen form. c – Equilibrium concentration of serum albumin in solution in mg/g; m – amount of adsorbed albumin in mg/g.

hydrogen form. This could be foreseen from our earlier data on the sorption of peptides [2]. With increased distance between the amino and carboxyl groups in the series amino acid–dipeptide–tripeptide, the sorption capacity for dipolar ions of the sulforesin in the sodium form increases, because of the weakening of the effect of electrostatic repulsion of ions and ionite. Thus, there is a criterion for estimating the distance between amino and carboxyl groups in globular proteins. The effect of the negative charge of the carboxyl group on the sorption of proteins by sulforesins in the salt form can be decreased by different processes. It is known that in water-acetone solution the degree of dissociation of the carboxyl group of amino acids and proteins is decreased compared to water solutions [3]. In agreement with this (Table 2), the sorption capacity for proteins of sulforesins in the salt form from water-acetone solutions greatly surpasses the sorption capacity of proteins from water solution.

Another process for removing the electrostatic repulsion in the sorption of proteins by sulforesins in the sodium form consists in screening the charges of the ion with increasing ionic strength of the solution in accord with the Debye-Hückel theory of strong electrolytes. As is seen from Fig. 3, when the concentration of the phosphate buffer solution (pH 7.4) is increased, the sorption capacity for proteins rises. Further fall in sorption capacity for albumin and γ-globulin is connected with the competing action of the sodium ion.

Although sorption of proteins by sulforesins is also a process of ionic sorption, yet the adsorption isotherms for proteins should differ from the equation for the isotherms of ion exchange since the process described by reaction (1) is not an ion exchange. There is no difficulty in deriving by thermodynamic methods an equation for

TABLE 1

Sorption Capacity for Proteins of SBS Resin in Hydrogen and Sodium Forms

Protein	Sorption capacity, mg/g	
	H form of resin	Na form of resin
γ-Globulin	171	0
Serum albumin	145	0
Ovalbumin	96	0
Insulin	125	30
Adrenocorticotropic hormone	117	63
Myogen	47	26
Chymotrypsinogen	90	105

TABLE 2

Sorption Capacity for Proteins of Sulforesin SBS in the Sodium Form from Water and Water-Acetone Solutions

Protein	Sorption capacity mg/g		Acetone, %
	from water solution	from water-acetone solution	
Serum albumin	0	97	40
γ-Globulin	0	132	20
Insulin	30	94	75

TABLE 3

Sorption Capacity for Proteins of Carboxyl Resin KMT in the Hydrogen and Sodium Forms

Protein	Sorption capacity, mg/g	
	H form of resin	Na form of resin
Serum albumin	120	0
Insulin	156	16
γ-Globulin	140	40
Adrenocorticotropic hormone	160	77
Chymotrypsinogen	284	136

the adsorption isotherm for the process of adsorption of dipolar ions [4] with the form of the Langmuir equation:

$$m = \frac{ac}{1 + bc}, \tag{2}$$

where m is the amount of adsorbed protein, c is the concentration of protein in solution, and a and b are constants.

The experimentally determined isotherm for the adsorption of serum albumin by the sulforesin SBS in the hydrogen form (Fig. 4) actually has this appearance. Carboxyl resins in the hydrogen form usually cannot adsorb ions from neutral or acid solutions. On the basis of our experimental investigations we have expressed the idea [5]

that sorption of ions by carboxyl resins in the hydrogen form proceeds very slowly because of the very small rate of diffusion of the desorbed ions from the particle of sorbent into the solution. When proteins are adsorbed there is no desorption of hydrogen ions from the cation exchange resin [Equation (1)] and so in the case of carboxyl resins the part of the process which most slows the sorption process is absent. In agreement with this, carboxyl resins in the hydrogen form adsorb large amounts of proteins from neutral solutions of 0.001 M phosphate buffer, pH 7.4 (Table 3). The sorption capacity was determined by the static method on resin KMT with a swelling coefficient in the hydrogen form of 3.6. The grinding of the resin was carried out in the same way as was described for the sulforesin.

The sorption capacity for proteins of the carboxyl resin in the sodium form was determined on specially synthesized resin KMT obtained with a large amount of bridge forming agent. This variety of the resin in the sodium form had the same swelling coefficient (3.6) as the preceding type of the resin in the hydrogen form. In agreement with the theory of sorption of dipolar ions [2] the carboxyl resin in the sodium form adsorbed considerably less protein than did the carboxyl resin in the hydrogen form. The ability of the carboxyl resin in the hydrogen form to adsorb adrenocorticotropic hormone was observed earlier [6].

SUMMARY

It has been established that sorption of proteins by cation exchange resins proceeds in accordance with the laws of adsorption of dipolar ions. Proteins are adsorbed with greater capacity by cationites in the hydrogen form compared to adsorption by cationites in the sodium form. Sorption of proteins by cationites in the salt form increases on change to a water-acetone solution or with increased ionic strength of the solution. Sorption of proteins by carboxyl resins in the hydrogen form occurs in accordance with the diffusion of hydrogen ions from the resin particles which is the slowest step in the sorption of ions by carboxyl resins in the hydrogen form from neutral solutions.

LITERATURE CITED

[1] G.V. Samsonov and M.D. Faddeeva, Biokhimiya 21, 403 (1956).

[2] G.V. Samsonov and N.P. Kuznetsova, Doklady Akad. Nauk SSSR 115, 351 (1957).*

[3] K. Linderstrom-Lang, Z. Physiol. Chem. 173, 32 (1928).

[4] G.V. Samsonov and N.P. Kuznetsova, Kolloidnyi Zh. 20, 209 (1958).*

[5] G.V. Samsonov and A.G. Sirota, Contributions to the 2nd All-Union Conference on Antibiotics [in Russian] (1957).

[6] H.B.F. Dixon and M.P. Stack-Dunne, Biochem. J. 61, 483 (1955).

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*Original Russian pagination. See C.B. translation.

THE PROBLEM OF THE NATURE AND ORIGIN OF THE WATER-SOLUBLE PIGMENT COMPLEX FORMED BY BIRCH CHAGI FUNGUS

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In the Botanical Institute of the Academy of Sciences USSR and the First Leningrad Medical Institute a complex study has been carried out for medical purposes on the chagi, the nonfertile form of the birch fungus Inonotus obliquus (Pers.) Pil. The idea was expressed that the medicinal action of chagi preparations is connected with its precipitation of a pigment complex [1].

In our preceding work we showed [2] that the pigment complex obtained from a water extract of chagi by precipitation with HCl formed by hydrolysis a series of aromatic hydroxyacids among which we were able to isolate and identify syringic, vanillic, and parahydroxybenzoic acids. These acids were also found to a slight extent in the free state, that is, before hydrolysis of the complex. However, the total amount of ether-soluble compounds in the free state was small and comprised only about 0.5-1.0% of the dry complex residue.

After hydrolysis of the complex, the amount of ether-soluble compounds rose to 15-17%. This indicates that the precipitated chagi-pigment complex is formed from aromatic compounds.

Our problem was to consider in more detail the source of the amorphous colored substances accumulated by chagi in great quantity, up to 16% of the dry weight of the mycelia. A solution of this problem could serve as a basis for working out a rational nutritive medium for the formation of the pigment complex of chagi in artificial cultures.

For an explanation of this problem we must turn most of all to the biology of this birch fungus.

It is known that under the action of tree destroying fungi the breakdown of the wood can occur differently. One group of fungi which belong to the so-called white rot, or, more correctly, the corrosion type of rots, destroy lignin more strongly than cellulose, while another group of fungi which belong to the brown rot or destructive type of rot first destroy cellulose [3]. The destructive action of fungi belonging to the first group, to which chagi also belong is shown chiefly in the destruction of the bond between lignin and cellulose and in splitting the lignin molecule into simpler compounds. The process of destruction of the lignin molecule is accompanied by replacement of the methoxyl group by carboxyl [4, 5].

Hence we can assume that the water-soluble, colored, amorphous substances which make up the organic chagi complex are the result of a secondary synthesis carried out by the fungus with fragments of the lignin molecule. Some of the properties of the complex, its amorphous nature, ability to precipitate when the pH reaches 2.5-3.0, great cation capacity, ability to change color under the influence of alkali, all lead us to suppose that the pigment complex belongs to the highly polymerized substances of the type of humic acid. The basic difference of the complex from humic acid is that it dissolves in water and contains almost no nitrogen.

Many studies devoted to explaining the structure and origin of humic acids have indicated a great role for microorganisms and fungi in their formation [6, 7]. The decomposition of plant residues by fungi and microorganisms involves biochemical oxidation of lignin. According to Trusov [8] this process proceeds in stages: hydrolytic splitting of aromatic compounds, oxidation of the resulting simple aromatic compounds with formation of quinones, and condensation of the quinones and their transformation into dark colored substances.

Starting from these ideas, we suggest that the precipitated complex should contain aromatic compounds analogous to those of lignin and humic acid and be less methoxylated than lignin and more methoxylated than humic acid, since in humic acid the oxidation and condensation processes of the aromatic nuclei should be deeper because of enzymatic action by the different microorganisms.

EXPERIMENTAL

In the experiments we took a water extract of the chagi fungi the pigment complex of which was precipitated by hydrochloric acid; birch lignin isolated by the alkali method; birch wood, healthy and infected by chagi; water extract of cork layer and bark of the birch; peat humic acid isolated by the method of Turin; humic acid from black earth obtained from the Soil Institute of the Academy of Sciences USSR, from Prof. Kononova. The water extracts of chagi, bark and cork layer were obtained by five-hour standing of the defatted dry material at 80-85°. Elementary composition (carbon and hydrogen) were determined by the Liebig method, combustion, and the methoxyl groups by the method of Fiebok and Schwappach [17].

For the study of the composition of the compounds which we investigated we used the method of alkali-nitrobenzene oxidation according to Freudenberg [9] and hydrolysis with 10-12% HCl at a pressure of 1.5 atm. for two hours [2]. Using the alkali nitrobenzene oxidation we showed the presence in all the samples of vanillin and syringic aldehyde. However, the results which more fully characterized our compounds were obtained by hydrochloric acid hydrolysis. The hydrochloric acid hydrolysate was repeatedly treated with ether. The ether fraction was separated according to Gatterman-Wieland [10] into the chief groups of substances. The acid fraction of the ether-soluble hydrolysis products was studied by paper chromatography and on a column.

The elementary composition of the precipitated chagi complex, lignin, and humic acid is given in Table 1.

As assumed, the number of methoxyl groups in the precipitated chagi complex is almost three times less than in lignin. This also agrees with the literature data on the demethoxylating action of wood destroying fungi [3]. Humic acid contains the least quantity of methoxyl groups.

TABLE 1

Elementary Composition of the Compounds Studied in % of Dry Weight

Substance studied	C	H	O (by difference)	OCH_3	N
Water-soluble precipitated chagi pigment complex	53.28	4.45	42.2	5.0	–
Birch lignin	61.50	5.55	32.9	15.7	–
Peat humic acid	57.50	5.90	34.1	1.2	2.50

Study of the hydrolysis products of the above-mentioned compounds showed that the precipitated chagi complex forms the greatest quantity of ether-soluble compounds. This indicates that the precipitated pigment complex is the most reactive compound with less strong bonds than lignin and humic acid. The results of the experiment are given in Table 2.

The smallest amount of ether-soluble compounds was obtained from the hydrolysis of healthy wood but in the wood attacked by fungus the amount of these compounds increased six times. Acid compounds predominated in the ether fraction of the hydrolysate: in the chagi pigment complex they comprised 90%, in lignin 86%, in humic acid 78% in birch cork 65%, and in birch bark only 33%. The greatest quantity of phenolic compounds occurred in bark and cork layer.

Study of the ether-soluble products of hydrolysis by paper chromatography in the system chloroform – acetic acid – water (50 : 25 : 25) showed that in all cases except for the bark we had similar chromatograms in which the spots had the same R_f values and the same color with diazo reagents. We always found syringic, vanillic and parahydroxybenzoic acids.

The difference between the chromatograms of the precipitated chagi complex and the other substances is

TABLE 2

Characteristics of the Ether-Soluble Compounds Formed by Hydrolysis, Given in % of Starting Dry Substance

Object studied	Acids	Phenols	Bases	Neutral substances	Total amount of ether-soluble substances
Precipitated chagi complex	16.20	0.50	0.08	0.30	17.09
Peat humic acid	5.52	1.00	0.07	0.10	7.00
Black earth humic acid	3.92	0.50	0.02	0.20	4.80
Birch lignin	6.41	0.80	0.10	0.10	7.41
Healthy birch wood	1.39	0.50	0.20	0.40	2.49
Birch wood infected with fungus	7.19	0.80	0.10	0.30	8.30
Cork layer (water extract)	5.20	2.0	0.10	0.80	8.10
Birch bark (water extract)	1.20	0.80	0.20	1.40	3.6

found chiefly in the acids which move directly at the front of the solvent and have a dark-brown fluorescence. These acids are seen in greatest amount in the hydrolysis of the precipitated chagi complex and are found in only slight quantity in the hydrolysate from humic acid. We carried out a quantitative estimation and separation of these compounds on a silica gel column by the method of [2] which we described earlier (Table 3).

Table 3 shows that the precipitated pigment complex from chagi is characterized by a high content of syringic acid. Vanillic and parahydroxybenzoic acids are found in the greatest amounts in the hydrolysate from humic acid. This is a further indication that this compound is less methoxylated than the chagi complex. Fraction A obtained from the precipitated chagi pigment complex is qualitatively different from fraction A obtained from the other substances. For fraction A obtained from the hydrolysate of precipitated chagi complex (independent of the source of the fungus) the presence of acids with an intense dark brown fluorescence is characteristic. These acids make up 35-40% of the weight of fraction A. According to preliminary data we can assume that they consist of a mixture of methoxylated derivatives of hydroxycinnamic acid in the cis form. The action of alkali decomposes these acids, forming syringic and vanillic acids.

The results of our experiments agree with the ideas of many investigators [11, 12, 13] that lignin contains phenylpropane units. For lignin from deciduous species two structural units are characteristically present: coniferyl and sinapic alcohols. We can assume that under the action of fungus enzymes on lignin there occurs oxidation and the molecule splits to the basic phenylpropane units which are oxidized to the corresponding acids. It is possible that these acids or their derivatives are the basic nucleus of the chagi pigment complex joined by ester links or by the Freudenberg dehydropolymer type [14]. According to Mentzer [15] phenylpropane derivatives during polymerization can form different compounds of the type of lignan and lignin. The presence in fraction A of acids of this type would explain their strong physiological action.

Manskaya [11] and other investigators [16] inclined to the view that the lignin of wood could be broken down by fungi to simple monomeric units. Kononova [6] also considered that lignin could share in the formation of humic acid only after it had decomposed to soluble phenolic units.

The question arises whether all the molecules of the complex have the same structure or only a part of them are formed on the basis of the phenylpropane derivatives. In experiments which we carried out on the repeated hydrolysis of the pigment complex we succeeded in showing that with each successive hydrolysis the same compound was split out.

In Table 4 we give the results of two experiments on the repeated hydrolysis of the precipitate. In the first experiment the unhydrolyzed residue of the pigment complex after hydrochloric acid hydrolysis was dissolved in alkali and submitted to alkaline hydrolysis under the same conditions (two hours under a pressure of 1.5 atm) after which the remaining insoluble residue was again hydrolyzed by HCl. In the second experiment both the first and second hydrolyses were carried out with 20% potassium hydroxide (two hours, 1.5 atm).

In the double alkaline hydrolysis we obtained a yield of ether-soluble compounds of 36% of the precipitate used in the experiment. The compounds which made up fraction A were then qualitatively different from the

TABLE 3

Content of Aromatic Acids in the Ether Fraction of the Hydrolysate, Given in % of Starting Dry Substance

Substance taken for hydrolysis	Fraction from silica gel column					Total of substances eluted from column	Ratio of syringic to vanillic acids
	A	B. pigment	C. syringic acid	D. vanillic acid	E. parahydroxybenzoic acid		
Peat humic acid	17.40	16.23	6.67	13.33	8.70	52.33	0.5 :1
Cork layer of birch	25.10	7.10	6.80	13.04	6.70	58.74	0.52:1
Birch lignin	28.34	13.97	9.11	3.97	5.26	60.65	2.3 :1
Precipitated chagi complex:							
from ordinary birch (Leningrad region)	28.16	6.20	16.8	6.35	2.00	60.0	2.6 :1
from far eastern yellow birch	41.31	1.90	16.68	4.28	2.04	66.21	3.8 :1
from ordinary birch (Lake Baikal region)	30.68	3.63	17.04	9.31	1.00	61.65	1.8 :1
from alders	35.60	2.47	14.24	6.85	1.80	60.96	2.09:1

TABLE 4

Formation of Aromatic Acids in Repeated Hydrolysis of Chagi Pigment Complex, Given in % Dry Weight of Ether-Soluble Compounds from the Hydrolysis

Nature of the hydrolysis	Yield of ether-soluble compounds, in % dry weight of complex	Fractions of silica gel column					Total of eluted substances
		A	B. pigment	C. syringic acid	D. vanillic acid	E. parahydroxybenzoic acid	
First acid	16.9	25.17	2.39	17.11	8.56	1.2	54.35
First alkali	5.8	1.90	2.48	14.30	4.60	1.60	24.88
Second acid	2.7	1.82	0	14.48	3.05	2.13	21.48
First alkali	20.30	5.40	5.16	18.02	6.50	1.53	36.58
Second alkali	16.13	3.94	3.94	20.86	7.51	–	36

compounds in fraction A with hydrochloric acid hydrolysis. The acids which gave a dark brown fluorescence were decomposed in the alkaline hydrolysis so that we did not observe them in lignin obtained by the alkali process. Also, in the composition of the ether-soluble compounds we found a greater amount of strongly oxidized products than in hydrochloric acid hydrolysis; these were not eluted at the given pH from the silica gel column.

Fraction B of the silica gel column contained pigment whose quantity depended strongly on duration and character of the hydrolysis, from which we can conclude that these pigments form during hydrolysis because of condensation of aromatic rings. Fractions C, D and E contained, respectively, syringic, vanillic and parahydroxybenzoic acids.

Among the aromatic compounds adsorbed on the silica gel column we observed protocatechic and pyrogallic acids which perhaps formed in the process of further oxidation of syringic and vanillic acids.

All these facts indicate that the amorphous water-soluble pigment complex formed by chagi fungus is a product of secondary synthesis from the fragments of the lignin molecule. At the basis of the structure of the complex there is evidently oxidation and partial demethylation of the monomeric structural units of birch lignin so that this complex can be related to high polymer products of the type of humic acid. In physical and chemical properties (solubility in water, number of functional groups) it occupies an intermediate position between the fully methoxylated molecule of lignin and the almost completely demethoxylated humic acids.

The role of the pigment complex and its physiological significance for the fungus is still not clear. We can assume that with the properties of the complex to bind and take out great quantities of ash elements which accumulate in the fungus mycelia during their long life, the pigment complex can play a role in the metabolism of the fungus. The water-soluble pigment complex can easily be oxidized so that it can take part in oxidation-reduction processes which proceed in the fungus mycelia.

On the basis of all the chemical and physical properties of the water-soluble precipitated pigment complex of chagi we can safely conclude that this compound is a high-polymer acid of the type of humic acid. We suggest the name "chagic acid" for it.

SUMMARY

Investigation of the products of hydrolysis of some natural products of aromatic character (birch lignin, humic acid, the water-soluble pigment complex formed by chagi fungus, etc.) by paper and column chromatography we have found the same aromatic hydroxyacids (syringic, vanillic, parahydroxybenzoic).

The distinct sign which characterizes the composition of the chagi pigment complex is the presence of aromatic acids which have a dark brown fluorescence. These acids are not stable; they are decomposed by alkali yielding syringic and vanillic acids.

The water-soluble pigment complex of chagi in the number of its functional groups occupies an intermediate position between lignin and humic acid. We propose the name chagic acid for the compound.

On the basis of the results of the investigation we suggest that the water-soluble precipitated chagi pigment complex is synthesized by the fungus from partly demethoxylated and oxidized structural units of birch lignin.

LITERATURE CITED

[1] P.A. Yakimov, P.K. Bulatov and M.P. Berezina, Vestn. AN SSSR 4, 88 (1957).

[2] E.V. Lovyagina, A.N. Shivrina and E.G. Platonova, Biokhimiya 23, 41 (1958).*

[3] S.I. Vanin, Wood Production [in Russian] (Goslesbumizdat, Moscow-Leningrad, 1949).

[4] P.N. Odintsov and Z.N. Kreitsberg, Izv. AN Latv SSR 1 (78), 67 (1954).

[5] P.N. Odintsov and Z.N. Kreitsberg, Izv. AN Latv SSR 9 (86), 97 (1954).

[6] M.M. Kononova, Problems of Soil Humus and Contemporary Problems of Its Study [in Russian] (Izd. AN SSSR, Moscow, 1953).

*See English translation.

[7] M.M. Kononova, Pochvovedenie 12, 45 (1955).

[8] A.G. Trusov, Materials for the Study of Soil Humus, part 1 [in Russian] (Petrograd, 1917).

[9] K. Freudenberg, W. Lautsch and K. Engler, Ber. Dtsch. Chem. Ges. 73, 167 (1940).

[10] L.I. Gatterman and H. Wieland, Practical Handbook of Organic Chemistry [in Russian] (Goskhimizdat, Moscow-Leningrad, 1948).

[11] S.M. Manskaya, Uspekhi Sovrem. Biol. 14, 19 (1957).

[12] K. Kratzl and G. Billek, Holzforschung 7, 66 (1953).

[13] K. Freudenberg, Forsch. der Chem. organisch. Naturst. 11, 43 (1954).

[14] K. Freudenberg, Z. Pflanzenernähr., Dung. Bodenkunde 69 (114), 1 (1955).

[15] C. Mentzer, Bull. Soc. chim. biol. 36, 1225 (1954).

[16] S.R. Bose, Ergebn. Enzymforsch. 8, 267 (1939).

[17] K. Bauer, Analysis of Organic Compounds [in Russian] (Izd. Inostr. Lit., Moscow, 1953).

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INTERMEDIATE NUCLEOTIDE METABOLISM IN BONE MARROW IN NORMAL SUBJECTS AND AFTER X-RAY IRRADIATION

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It is known that bone marrow is one of the organs with very intense nucleic acid metabolism. At the same time, it is one of the organs which is severely damaged by radiation sickness [1, 2]. A number of authors have shown that in bone marrow the metabolism of desoxyribonucleic acid (DNA) and ribonucleic acid (RNA) after x-ray irradiation is damaged in the same way, except that the RNA metabolism is damaged somewhat less than that of DNA.

The metabolism of low molecular weight nucleotides in bone marrow in normal subjects and after x-ray irradiation has not been sufficiently studied. In particular, the literature contains nothing concerning the action of ionizing radiation on the content of the different purine and pyrimidine fractions in bone marrow.

In the present work we give the results of some investigations on this problem and data on the splitting of DNA in bone marrow homogenates in normal subjects and after irradiation.

EXPERIMENTAL

In order to avoid errors from individual variations in comparing irradiated and unirradiated bone marrow we took samples from the same animal. The right hindleg of a rabbit was irradiated and the rest of the body was shielded with a lead sheet 5 mm thick.

The source of the x-ray irradiation was an RUP tube, 180 kv, 15 ma, $\lambda = 0.15$ A; filters: Cu = 0.5 mm and Al = 0.75 mm. Duration of irradiation 21 minutes; dose 2000 r. Two days after the irradiation the animal was decapitated and exsanguinated, after which the bone marrow was rapidly removed (in a cold room) separately from the right and left hindlegs and was homogenized.

In a weight of homogenate we determined the content of preformed nucleotides and also the enzyme activity.

For the determination of the content of preformed nucleotide in bone marrow we took a sample of 0.3-0.5 g of bone marrow homogenate. To this we added 10 ml of H_2O and quickly brought the mixture to boiling, then added 20 ml of 95% ethanol and again brought the mixture to boiling.

After the mixture was cooled, it was filtered, the precipitate on the filter was washed with 60% alcohol, and the filtrates were evaporated at temperatures not above 40°. The dry residue was dissolved in a small amount of water and placed on chromatographic paper. The original spot on the chromatogram was a line 4 cm long.

For the chromatography we used Leningrad "slow" paper, first washed with 25% formic acid. We used descending chromatography. As a solvent we used a mixture of 95% ethanol + anhydrous n-butanol + 1 M acetate-ammonia buffer at pH 3.7-3.8 in the ratio 4 : 1 : 2 [3]. The chromatogram was 45 cm long. When the solvent front reached the lower edge of the chromatogram, the latter was removed from the chamber, dried, and again placed in the chamber and the solvent passed for a second time to the lower edge of the chromatogram. The dried chromatogram was viewed in the ultrachemiscope [4]. The spots which absorbed ultraviolet light were outlined in pencil. The water eluate of the spots seen in the ultrachemiscope was studied in an SF-4 spectrophotometer.

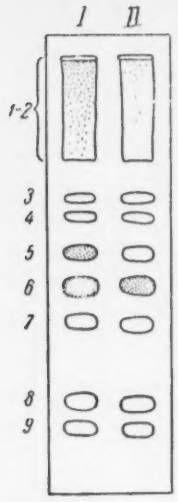

Fig. 1. Typical distribution on the chromatogram of low molecular weight purine and pyrimidine compounds contained in normal bone marrow and marrow after irradiation: I) normal; II) two days after local irradiation with a dose of 2000 r. Figures on the left – fraction numbers.

By this method we detected in bone marrow tissue various low molecular weight substances with absorption maxima characteristic of nuclein bases (in most cases at 250 or 260 mμ). The position of these fractions on the chromatogram (Fig. 1) characteristic for nucleotides (middle part of the chromatogram) showed that the relative content of fraction 6 was the greatest.

In parallel chromatograms with labeling of the different nucleotides it was shown that fraction 6 corresponded to muscle adenylic acid, fractions 8 and 9 corresponded to free nuclein bases.

The amount of low molecular weight purine and pyrimidine fractions contained in bone marrow is shown in Table 1. Spectrophotometry of the solutions was carried out on water eluates of the spots (5 ml H_2O). The resulting values were corrected for absorption of the extract by the paper (E = 0.025) after which the results were calculated per g of tissue.

The table gives the data for two typical experiments.

On the basis of the data of Table 1 we can calculate that two days after localized x-ray irradiation of 2000 r the content of low molecular weight purine and pyrimidine compounds in bone marrow has fallen on the average by 60%.

Enzymatic splitting of DNA in bone marrow homogenates was studied in the following buffer solutions: orthophosphate, pyrophosphate, tris buffer* and glycine with 0.3 M NaCl. In each sample we took 20 mg of DNA (dissolved in 0.85 ml of 0.05 N NaOH, pH 7.0) + 2 ml 0.3 M buffer solution with pH 7.0 + 0.3 g of bone marrow.

We used a commercial purified DNA preparation for the investigation. We ran parallel control experiments without adding bone marrow homogenate and without adding DNA. After the mixtures had been incubated in a thermostat at 37° for an hour, the sample was quickly heated to boiling, then to each sample was added 4 ml of 35% ethanol and the sample was again heated to boiling; after it had cooled, the sample was filtered.

The filtrates were put on the chromatogram paper and analyzed in the same way as the preformed low molecular weight purine and pyrimidine compounds (see above).

One of the problems of this study of the separations was the effect of the buffer solutions on splitting of DNA in bone marrow. In this connection, we did not consider that it was satisfactory to determine the total amount of low molecular weight compounds split from DNA in different buffer solutions, since one component might be split from DNA more completely in one buffer and another component in another buffer, yet the total quantity of compounds split out would be the same.

The effect of buffer solutions on the splitting out of the different compounds from DNA was shown previously by us in experiments with liver [5].

Figure 2 shows the distribution on the chromatogram of the products of enzymatic splitting of DNA by bone marrow homogenate as seen in the ultrachemiscope.

The water eluates of the spots seen in the ultrachemiscope were studied in the SF-4 spectrophotometer. Absorption maxima for the fractions lay within the limits 250-265 mμ (most often at 250 or 260 mμ).

The quantity of products of DNA splitting, expressed in extinction at the maximum point, is shown in Table 2.

We placed 0.4 ml of filtrate on the chromatogram. Spectrophotometric study was carried out on the eluate from two spots with 5 ml of H_2O. The resulting values were corrected for absorption of the extracts from the paper (usually E = 0.050).

*Tris(hydroxymethyl)aminomethane.

TABLE 1

Amount of Purine and Pyrimidine Compounds (Fractions) Contained in Bone Marrow of Rabbits, Normal and Two Days after Localized X-Ray Irradiation With 2000 r

Experimental conditions	Fraction number						
	1-2	3	4	5	6	7	9
Normal	0.328	0.110	0.102	0.418	2.077	0.121	0.469
Irradiated	0.030	0.034	0.038	0.172	0.710	0.106	0.366
Irradiated in % of normal	9.1	30.9	37.2	41.1	34.2	87.5	76.7

TABLE 2

Quantity of Products (Fractions) Formed in the Enzymatic Splitting of DNA in Bone Marrow Homogenates With Different Buffer Solutions (quantity of the fractions expressed in value of extinction at the maximum point)

Fraction No.	Normal				Two days after irradiation with 2000 r			
	glycine buffer solution	orthophosphate buffer solution	pyrophosphate buffer solution	tris buffer solution	glycine buffer solution	orthophosphate buffer solution	pyrophosphate buffer solution	tris buffer solution
2	0.075	0.153	0.115	0.198	0.045	0.153	0.212	0.047
3	0.093	0.188	0.165	0.260	0.094	0.169	0.127	0.144
5	0.047	0.699	0.610	0.810	0.062	0.547	0.419	0.462
6	0.039	0.859	0.496	0.610	0.029	0.668	0.319	0.387
7	0.027	0.281	0.237	0.407	0.033	0.245	0.165	0.268
8	0.190	0.074	0.011	0.000	0.081	0.028	0.038	0.000
Total extinction	0.471	2.254	1.634	2.285	0.344	1.810	1.280	1.308
In % of normal	–	–	–	–	73.1	80.2	78.3	57.2

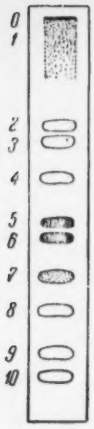

Fig. 2. Typical chromatographic separation of the products of enzymatic splitting of DNA in bone marrow: O) starting position; 1-10) products (fractions) seen in the ultrachemiscope.

The table was drawn from the data of two typical experiments. The same experiments were carried out with a longer incubation period (2-3 hours instead of 1 hour). Analogous results were obtained. In all, ten experiments with each variation were run in this series.

The data of Table 2 show that enzymatic formation of most of the fractions from DNA in bone marrow homogenates occurs with practically the same intensity in tris buffer solution as in phosphate buffers.* X-ray irradiation in vivo suppresses the enzymatic splitting of DNA by bone marrow homogenate in different buffer solutions by 28%.

The question arises as to whether there is any obstacle to the study of enzymatic splitting of nucleic acids from those which are present already in bone marrow homogenate. However, control experiments without adding DNA showed that the amount of low molecular weight purine and pyrimidine compounds formed without adding DNA was slight under these experimental conditions and therefore could not offer any obstacle. The total amount of low molecular weight fractions formed from DNA was calculated to be about 20-30 times greater than the amount of low molecular weight nucleotides which were introduced into the experimental samples as part of the bone marrow homogenate. The calculation can be carried out on the basis of data given in the first section of our work.

We judge from the reaction with $AgNO_3$ that even after three-hour incubation of the reaction mixture with pyrophosphate buffer solution, the pyrophosphate in it is split only slightly.

DISCUSSION OF RESULTS

In the present work we have studied the action of x-ray irradiation on intermediate metabolism of those low molecular weight purines and pyrimidines which are soluble in 60% alcohol. The chief members of this group are the nucleotide monophosphates, and also free purine and pyrimidine bases.

At present the literature reports on the nucleotide content in irradiated tissues can be divided into two directly contradictory groups.

In the first group occurs the work of Ererra, Davidson and Kanazir [6-9] which indicates an increased nucleotide content in tissues irradiated by x-rays and ultraviolet light. On the basis of these data it can be concluded that the suppression of biosynthesis of nucleic acids after irradiation is not caused by suppression of nucleotide synthesis or their lower content in the tissues, but by suppression (blocking) of the process of polymerization of nucleotides into nucleic acids.

Some authors [10] found after x-ray irradiation of animals an increase in acid soluble substances which reacted with diphenylamine in lymphoid tissue, which they explained not as a blocking of the synthesis of DNA from nucleotides, but as an increased catabolism of DNA.

In the second group belongs the work which indicates a decrease in nucleotide content in irradiated tissue.

Thus, Hickman and Ashwell showed a decreased content of adenylic acid in the spleen after x-ray irradiation [11].

The lowered total content of extractable acids and substances which absorb ultraviolet light in bone marrow cells after x-ray irradiation was recently shown by the cytochemical study of Brodskii and Suétina [12]; they did not separate their substances into fractions.

Becarevic et al. [13] also observed that suppression of DNA and RNA synthesis occurred in rat spleens after irradiation with 1500 r and was not accompanied by an increased content of low molecular weight nucleic acid derivatives, to judge by the total absorption at 260 mμ of the acid extracts from the spleen.

*As was shown in experiments with Kopylov, when $MgCl_2$ was added, the total splitting of DNA in bone marrow homogenates occurred much more intensively (on the average by 22%) in pyrophosphate than in orthophosphate and in tris buffer solutions. This shows that under some conditions the pyrophosphorylation reaction of DNA occurs.

We should mention the possible reasons for the decrease in amount of free nucleotides in bone marrow after irradiation and the results of this. With regard to the most often studied nucleotides of the muscle adenylic system, it is known that the deep and hardly reversible splitting which is accompanied by deamination of the nucleotides occurs only after the two terminal phosphate groups of ATP have split off. Therefore, we can assume that in bone marrow the decrease in amount of nucleotides after irradiation depends chiefly on their splitting, which occurs because of inhibition of oxidative phosphorylation and thus the conversion of nucleotide monophosphates into nucleotide di- and triphosphates.

Another possible reason for the decrease in quantity of nucleotides in bone marrow after irradiation is the change in permeability of bone marrow cells after irradiation.

The change in concentration of nucleotides in tissues after irradiation can have great physiological significance in three respects: a) inhibition of synthesis of nucleic acids, since nucleotides are the substrate for the synthesis of nucleic acids; b) inhibition of oxidative phosphorylation, since nucleotides share in the process of oxidative phosphorylation; c) inhibition of mitotic cell division, since separation of the chromosomes due to shortening of the spindle fibers during mitosis is evidently also produced by the action of nucleotides, like the mechanism of change of actomyosin fibers when they are acted upon by the free nucleotides of muscle [14].

Disturbance in the metabolism of free nucleotides along with disturbance of nucleic acid metabolism after irradiation by x-rays evidently can explain to a considerable extent the suppressing of cell division after x-ray irradiation.

In the literature up to now there has been no generally accepted opinion as to the effect of x-ray irradiation on DNA-ase activity. Manoilov and Semenov [15] have established that DNA-ase in vitro is comparatively easily inactivated with x-ray irradiation (1000 r).

However, Douglass and Day [16] showed that after total irradiation of the rat there was a sharp increase in specific DNA-ase activity in the spleen. It is true that as a result of the decreased weight of the spleen the total DNA-ase activity of the spleen increased only slightly.

According to the results of Kowlessar and co-workers [17] after x-ray irradiation of the rat there was an increased excretion of DNA-ase in the urine.

Our finding of the decrease in splitting of DNA in bone marrow homogenates evidently cannot be due to increased content of water in the tissues after irradiation, since, according to the results of Dietz [18] the amount of water in rabbit bone marrow two days after localized x-ray irradiation at a dose of 3000 r is even decreased somewhat (from 39 to 38.5%).

SUMMARY

The low molecular weight purine and pyrimidine compounds in bone marrow before and after irradiation were separated into eight fractions; we established the positions of these fractions under definite conditions of paper chromatography and also determined spectrophotometrically the content of these fractions.

We showed in parallel chromatograms with definite nucleotides that the sixth fractions which was present in the greatest amount of all the low molecular purine and pyrimidine compounds was adenylic acid.

The content of low molecular weight purine and pyrimidine fractions of bone marrow decreased in two days after localized x-ray irradiation with a dose of 2000 r. The total amount of the studied fractions in bone marrow of an irradiated leg decreased by about 60% compared to an unirradiated leg of the same animal.

We have suggested possible reasons for the decreased content of nucleotides in bone marrow after irradiation and the physiological significance of this.

Two days after localized x-ray irradiation of one leg of a rabbit with a dose of 2000 r, the enzymatic activity of the bone marrow of this leg, in splitting off from DNA of purine and pyrimidine compounds which are soluble in 60% alcohol, decreased differently in different buffer solutions: in tris buffer solution by 43%, in orthophosphate buffer solution, by 20%.

I express thanks to Z.A. Kuznetsova for help in the experimental work.

LITERATURE CITED

[1] I.I. Ivanov, V.S. Balabukha, E.R. Romantsev and T.A. Fedorova, Metabolism in Radiation Sickness [in Russian] (Medgiz, Moscow, 1956).

[2] Z.M. Bacq and P. Alexander, Fundamentals of Radiobiology (Butterworth Scientifiofic Publ., London, 1955).

[3] A.S. Spirin and A.N. Belozerskii, Doklady Akad. Nauk SSSR 113, 650 (1957).*

[4] E.M. Brumberg, Doklady Akad. Nauk SSSR 79, 885 (1950).

[5] G.A. Kritskii, Doklady Akad. Nauk SSSR 119, 336 (1958).**

[6] C.W. Bishop and J.W. Davidson, Brit. Radiol. 30, 367 (1957).

[7] M. Errera, Problems of Radiobiology [Russian translation] (Izd. IL, Moscow, 1956) p. 162.

[8] D. Kanazir, Arch. internat. physiol. 62, 299 (1954).

[9] D. Kanazir, Biochem. et biophys. acta 15, 592 (1954).

[10] S. Okada, E.K. Gordon, K. King, L.H. Hempelmann and B. Schlegel, Arch. Biochem. and Biophys. 70, 469, 76 (1957).

[11] J. Hickman and G. Ashwell, J. Biol. Chem. 205, 651 (1953).

[12] V.Ya. Brodskii and I.A. Suétina, Biofizika 3, 92 (1958).**

[13] A.D. Becarevic, K.J. Kacanski and D.D. Mancie, Experientia 12, 13 (1956).

[14] D. Mazia, Problems of Biophysics [Russian translation] (Izd. IL, Moscow, 1956) p. 189.

[15] S.E. Manoilov and L.R. Semenov, Doklady Akad. Nauk SSSR 71, 359 (1950).

[16] C.D. Douglass and P.L. Day, Proc. Soc. Exptl. Biol. and Med. 89, 616 (1955).

[17] O.D. Kowlessar, K.L. Altman and L.H. Hempelmann, Arch. Biochem. and Biophys. 52, 362 (1954).

[18] A.A. Dietz and B. Steinberg, Arch. Biochem. and Biophys. 23, 222 (1949).

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*Original Russian pagination. See C.B. translation.

**See English translation.

COMPLETE LABELING OF THE ORGANIC SUBSTANCES OF PLANTS BY RADIOACTIVE CARBON AS A METHOD FOR STUDYING METABOLIC DISTURBANCES

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In studying the mechanism of the action of various physical and chemical factors on living organisms one of the great problems is the investigation of those metabolic disturbances which depend on some given biological effect. It is possible to expect from this a change in the processes of formation or breakdown of specific metabolites, their distribution in organs and tissues, or the appearance of anomalous metabolites. The detection of these disturbances is often a very difficult problem, since we do not always know in which class of substances these changes occur, whether they will lead to a decrease in existing substances or the appearance of new metabolites, not to mention the fact that we do not have sufficiently sensitive methods for the qualitative and quantitative detection of many of the substances which are present in living organisms.

For studies in this direction, there is great promise in the present work on complete labeling with radioactive carbon of all the carbon-containing substances of the organism under investigation, and later study by radiochromatography.

The principle of the method is to create an equilibrium, with labeled C^{14}, of all the substances in the organism, so that later the substances or groups of substances which have been changed by the action under investigation can be isolated. In an experiment, half of the labeled organisms make up the control and show the normal state of the metabolites. The other half are submitted to some type of action (chemical agents: poisons, mutation-producing substances, auxins, carcinogens; physical agents: ionizing radiation, ultrasound, low temperature; infecting agents: viruses, bacterial infections, etc.). The experimental and control organisms are then fixed and fractionated under exactly the same conditions.

The fractions are studied by paper chromatography. The chromatograms are exposed on x-ray sensitive films. Visual comparison of the resulting radiochromatograms shows the appearance or disappearance of different metabolites as the result of one or another process, and measurement of the density of the darkened spots on the radiochromatogram, or the activity of the spots on the chromatogram itself, will give an idea of the decrease or increase in content of the various substances in the organism. Further identification of these substances can be carried out by the usual methods using colored reagents, determining the R_f of the observed spots as compared to standards, by studying the chromatogram in ultraviolet light, etc.

Carrying out total labeling of all the organic compounds with radioactive carbon is easiest in green plants by prolonged photosynthesis in an atmosphere which contains radioactive carbon dioxide [1, 2, 3, 4] etc. Total labeling of microorganisms can be produced by culturing them in a medium which contains glucose labeled with C^{14}. In the case of animals the problem is complicated and can be solved in different cases by feeding the animals on plants which are completely labeled with radioactive carbon.

In the present work we present data on working out this method for the example of the study of change in plant metabolism after the action of ionizing radiation. We start with the following requirements in the plants for the experiments: 1) all the organic substances in the plant should be labeled with C^{14}; 2) the specific activity of the substances should be sufficient for their detection by the radiochromatographic method and at the

same time should be small enough so that the metabolism does not vary from the normal; 3) the radioactivity of the different plants should vary within narrow limits so that it will be possible to collect equally radioactive sections of the plants for experiment and control.

To determine the conditions in which we will obtain complete labeling of plants, we have made preliminary studies with young tobacco, bean, poppy, sugar beet, corn and other plants.

We showed that for satisfactory labeling of such substances as mono-, di- and polysaccharides, four carbon dicarboxylic acids, aspartic and glutamic acids, and some others, it was sufficient to carry out photosynthesis in an atmosphere which contained 0.1% carbon dioxide with a total activity of 1.7 μC per g of fresh plant tissue, for a day or even for several hours. However, under these conditions many substances of secondary synthesis, such as chlorophyll or alkaloids, contained almost no C^{14}. In order to determine the time of photosynthesis needed for complete labeling of all the plant substances, we carried out experiments on alkaloid-producing plants to determine the activity of the isolated alkaloids.

In a study of the radioactive carbon which entered the alkaloid morphine in Papaver somniferum, we showed that only after 20-day cultivation of the mature plant in the light, in an atmosphere which contained 0.1% $C^{14}O_2$ with a total activity of 2 mC in 400 g of fresh plant tissue, did we obtain morphine with an activity of 30 μC/g. Radiochromatographic study of the isolated morphine gave only one spot on the radiochromatogram [2], which indicates the absence of radioactive contaminants in the isolated morphine.

When the growing plant absorbed radioactive carbon dioxide during 20-day photosynthesis with an activity of 300 μC per 100 g of fresh plant tissue, we obtained complete labeling of the plant with an activity of the different metabolites sufficient for their determination by radiochromatography. Calculation showed that during the period of the experiment (20 days) the dosage of the irradiated plants in β-particles reached 100-150 rep. Absorption of this amount of energy does not, as a rule, cause significant variation of metabolism from the normal.

The basic experiments were carried out with Voronezh-76 corn. The seeds were germinated on moist filter paper. From the three-day-old sprouts we gathered 100 plants with the same development and placed them for further growth in a water culture with a special support. After seven days the support with the plants was transferred to the photosynthesis chamber.

The chamber which we used had the dimensions 35 x 35 x 35 cm (Fig. 1), was made of organic glass and connected with a counter to calculate the concentration of radioactive CO_2 and an apparatus for maintaining the needed concentration of radioactive carbon dioxide in the chamber.

In 20 days of cultivation of the young corn plants in the chamber, labeled carbon dioxide was introduced ten times. During the whole experiment the 100 plants received 205 μC of radioactive carbon dioxide.

Photosynthesis went on for ten hours a day with illumination of the chamber through a water filter by two incandescent lamps of 500 w each. Interruption of photosynthesis at night permitted redistribution of the C^{14} from the primary products of photosynthesis to substances of secondary synthesis. The plants were grown on a Knop nutritive mixture. The small size of the chamber and the simplicity of its arrangement made the work with it satisfactory under laboratory conditions. The chamber was set up under a hood which permitted removal, without any danger to the workers, of the air when the concentration of radioactive CO_2 in the chamber after two days of photosynthesis had fallen to 0.003-0.001 μC per liter.

Selective measurements of the activity of separate plants after 20 days of culturing showed activity* on the order of 20,000 ± 3000 counts/min/mg of dry substance. Since in the experimental and control series we used 50 plants, this guaranteed homogeneity of labeling among the samples of experimental and control plants.

The experimental and control groups of fully labeled plants were placed in the moist chamber after which the experimental plants were irradiated with x-rays with a dosage of 800 ± 200 r per min. The full dose of irradiation of the roots was 10,000 r. Immediately after irradiation the experimental and control (unirradiated) plants, 50 in each group, were placed in an apparatus (Fig. 2) which permitted measurement of exosmosis of organic substances into distilled water from the roots of irradiated and unirradiated plants. Measurement of the activity of the water in which the roots were placed was carried out periodically, at first at intervals of one hour and then of five hours. The results of the measurement are given in the curve in Fig. 2.

*By activity is always meant the rate calculated in counts/min.

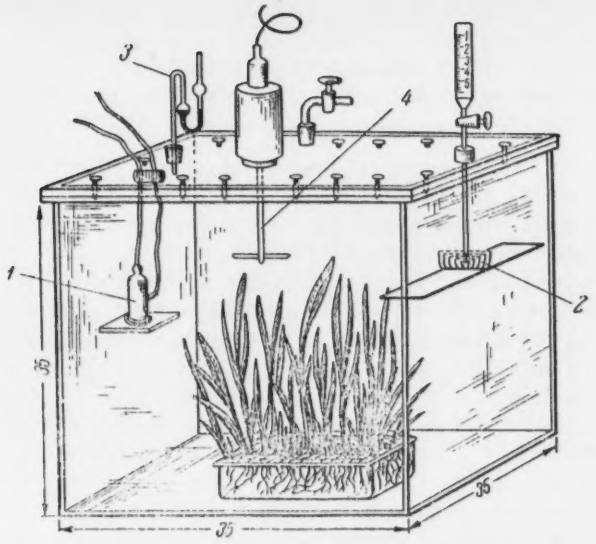

Fig. 1. Model of the laboratory chamber for obtaining plants labeled with C^{14}: 1) counter; 2) device for regulating the supply of $C^{14}O_2$ in the chamber; 3) manometer; 4) ventilator.

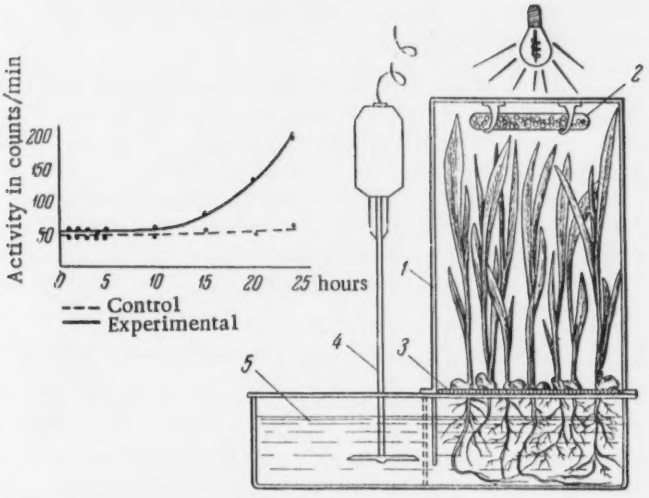

Fig. 2. Apparatus for determining exosmosis in plants: 1) plastic chamber; 2) tube with soda lime for absorbing $C^{14}O_2$; 3) support with plants; 4) electric stirrer; 5) place for taking samples.

It is interesting that in the first hour after irradiation we find almost no difference in exosmosis between control and experimental plants. The experiment lasted 24 hours. The plants during the experiment were illuminated. Measurement at the end of the 24-hour period showed a considerable difference between experimental and control plants. While the control plants showed only traces of exosmosis of organic substances, the irradiated plants showed clear evidence of their excretion.

Our method permitted not only establishment of a difference in amount of exosmosis of organic substances, but also a determination of their nature. For this purpose, after the roots of control and experimental plants had

Fig. 3. Radiochromatogram of exosmosive substances of irradiated plants: a) experiment; b) control. See text for identification of spots.

stood for 24 hours in the water, it was concentrated in a vacuum to a volume of 0.3 ml and placed on paper for chromatography. Chromatographic separation was carried out in a mixture of butanol-acetic acid-water (74:20:50). The chromatogram was dried and exposed on x-ray film XX for a month. The resulting radiochromatogram is shown in Fig. 3.

While the control plants show the presence of only small amounts of activity where the drop was applied (traces of protein substances?) the irradiated plants clearly show the presence of eight substances. When the chromatogram is viewed in ultraviolet light it shows sharp absorption at spot 1. The place on the chromatogram corresponding to spot 1 was cut out and the substance was eluted with water. Measurement of the absorption spectrum of the eluate showed an absorption maximum at 260 mμ, which showed the presence of a purine or pyrimidine base or their derivatives (nucleic acids). The small R_f of this spot makes the assumption likely that we are here dealing with a polynucleotide. To test this assumption we hydrolyzed the eluate for one hour at 100° with 70% hydrochloric acid. The resulting hydrolyzate after neutralization was filtered, concentrated in a vacuum, and submitted to chromatographic separation. A study of the radioactivity of the chromatogram and viewing it in ultraviolet light showed three spots with R_f = 0.23, 0.32, 0.50 (Fig. 4, spots 2, 3, 4) which correspond to guanine, cytosine, and adenine (uracil?).

Thus, we can consider it to be established that irradiated roots excrete a polynucleotide with R_f = 0.11, evidently a product of splitting of the plant nucleic acids. Spots 3, 4, 5 and 6 gave a reaction with ninhydrin and from R correspond to amino acids: aspartic acid, glutamic acid, threonine, methionine, and leucine. Spots 7 and 8 gave a yellow color with bromophenol and from R_f correspond to malic and succinic acids. In spot 2 a simple sugar is probably present (glucose R_f = 0.3), for which a color reaction gave a weakly positive result.

The substances excreted by the irradiated plants (organic acids, amino acids, polynucleotides) 24 hours after irradiation can be explained by the anomalous accumulation of these substances in the roots of the irradiated plants. Such an accumulation is possible because of the inhibition of utilization of these metabolites or because of the anomalous splitting of high-molecular-weight substances. We can also assume a disturbance in cell permeability. The excretion of polynucleotides with a lower molecular weight than the native nucleic acids, which do not migrate on the chromatogram with the solvent used, shows the splitting of nucleic acids in irradiated roots 24 hours after irradiation.

We also studied changes in the ratios of substances in the leaves and roots of irradiated plants.

At the end of the experiment with exosmosis, in the experimental and control plants we separated the leaves from the roots and after 24 hours these were fixed by liquid nitrogen. The frozen plant tissues were ground, dried in a deep vacuum, and extracted in a Soxhlet apparatus with dry ether to complete removal of the ether-soluble pigments and lipids.

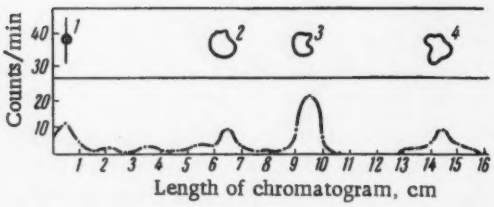

Fig. 4. Chromatogram of the hydrolyzate of substance 1.

The residue was extracted with 96% alcohol. After the alcohol-soluble fraction had been obtained, the rest of the substance was extracted with 4% trichloroacetic acid (TCA).

The solvents were removed from the separate fractions in a vacuum. TCA was removed by ether. The dry residues were dissolved in a standard amount of water and separated by paper chromatography with a mixture of butanol-acetic acid-water in the ratio 74:20:50. The chromatogram was exposed to x-ray film for 40 days.

Fig. 5a. Radiochromatogram of the alcohol fraction of roots and leaves of irradiated and control: a) experiment; b) control; I) leaves; II) roots. For identification of spots see text.

Fig. 5b. Radiochromatogram of TCA-fraction of roots and leaves of irradiated plants: a) experiment; b) control; I) leaves; II) roots. For identification of spots see text.

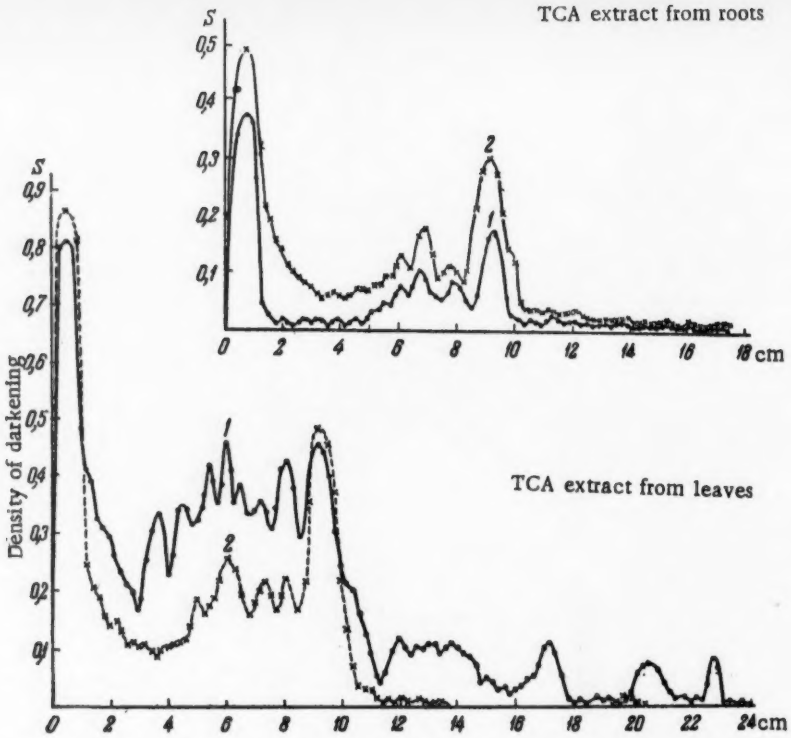

Fig. 6. Density of darkening of the radiochromatograms: 1) experimental; 2) control.

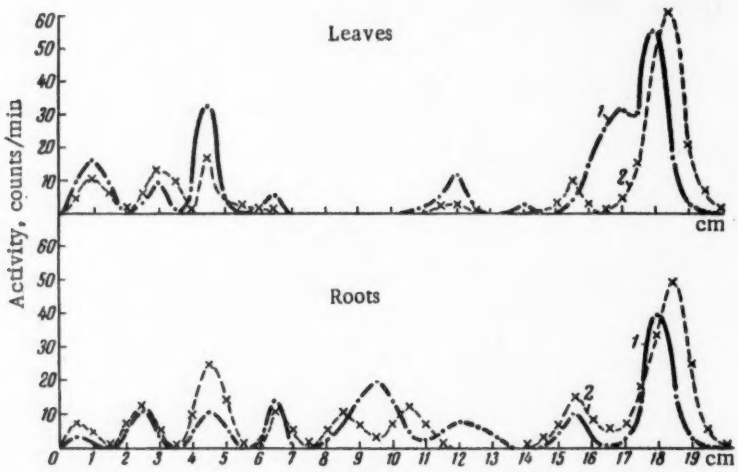

Fig. 7. Activity of radiochromatogram: 1) experimental; 2) control.

In the radiochromatogram (Fig. 5a) of the alcohol fraction we found 13 separate spots. Of these, 3, 5, 6, 7, and 8 gave a reaction with ninhydrin and probably contained amino acids. Spot 4 corresponded to glucose, which was confirmed by reaction with para-anisidine phosphate, giving a positive result in the control leaves and irradiated roots. As a whole, the radiochromatograms of the alcohol fraction of irradiated and control plants were similar to each other. There was a clear difference in the substances contained in spots 3 and 4 (according to R_f, aspartic and glutamic acids). They predominated in the irradiated roots compared to the normal ones, where

they were almost not found. They were also but little observed in irradiated leaves, while in normal leaves they were present in large amount. The same can be said for glucose and fructose, spots 4 and especially 3. The same redistribution between roots and leaves was also characteristic for the,as yet,unidentified substance which gave spot 2.

A considerable difference was found between irradiated and normal plants in the TCA extract (Fig. 5b); four of the twelve substances on the radiochromatogram (1, 2, 11, 12) appear only in the irradiated leaf. Of these, 1 and 2 show clearly in absorbing ultraviolet light, 11 and 12 are weakly developed by ninhydrin. The irradiated leaf is also characterized by the presence of large amounts of substances 9 and 10 (in the control there are traces, poorly visible in the reproduction of the radiochromatogram) and by an appreciable content of substance 7 compared to the control. Substance 7 absorbs ultraviolet light at 260 mμ and has $R_f = 0.31$. The analogous fractions from the roots are closer to each other and differ only in the smaller concentration of a number of metabolites in the irradiated root.

The difference in quantitative content is especially clearly seen in the results of photometric study of the radiochromatograms. In Fig. 6 we give the density of darkening measured on a microphotometer MF-4.

In the resulting radiochromatograms we note that a considerable amount of the substances extracted by TCA does not migrate in the solvent used and remains at the point of introduction. The use of the technique of fractionating radiochromatograms described by one of us earlier [5] allowed us to carry out a repeated chromatographic separation of the remaining spots in a 1% NaCl solution. The distribution of substances on the chromatogram was shown by a counter with a slot diaphragm. The resulting curves are given in Fig. 7. Analysis of these curves shows the similarity in composition of these fractions,in the irradiated and control plants.

SUMMARY

We have described a new method for studying disturbances of metabolism after the action of various physical, chemical, or biological factors. The method is based on use,for experimental organisms,of complete labeling by C^{14} and has been used for the study of disturbance of metabolism in plants under the influence of ionizing radiation. We have shown an increase in exosmosis of organic substances from the roots of irradiated plants,and determined the nature of these substances. We have shown a characteristic disturbance in metabolism in leaves and roots of irradiated plants. We have studied the radiochromatograms obtained from different fractions from fully C^{14}-labeled organisms, and this has allowed us to judge the changes in quantitative ratio of metabolites,and the disappearance or appearance of different substances under the influence of various agents as compared to the normal state.

LITERATURE CITED

[1] N. Skali, V. Chornii et al., Use of Radioactive Isotopes in Industry, Medicine and Agriculture [in Russian] (Izd. AN SSSR, Moscow, 1956) p. 594.

[2] A.M. Kuzin and B.I. Merenova (Tokarskaya),Biokhimiya 19, 616 (1954).

[3] V.I. Merenova (Tokarskaya), Biokhimiya 19, 6 (1954).

[4] W.C. Evans and M.W. Portridge, J. Pharmacy and Pharmacol. 6, 703 (1954).

[5] A.M. Kuzin and G.N. Saenko, Proceedings of the Commission on Analytical Chemistry, vol. IV [in Russian] (Izd. AN SSSR, Moscow) p. 461.

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THE CHANGES IN ELECTROPHORETIC AND IMMUNOLOGICAL PROPERTIES OF TISSUE AND SERUM PROTEINS OF RATS DURING PROTEIN DEPRIVATION, AND FOLLOWING CERTAIN TYPES OF LIVER AND KIDNEY DAMAGE

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It was shown in a previous communication [1] that certain protein fractions from brain, liver, and kidneys, whose electrophoretic mobilities corresponded with those of respective serum protein fractions, were also immunologically identical with those fractions. This held, in the first instance, for the kidney and liver albumins. Brain albumins were found to be only partially immunologically identical with the serum albumins. With respect to various tissue globulins it was established that, of those which showed identical electrophoretic mobilities with their serum counterparts, only a few were identical with the corresponding serum proteins immunologically. These data made it possible to draw certain conclusions about the origin of various tissue and serum proteins and about their interconvertibility.

It was considered of great importance to study the changes in the immunological properties of tissue and serum proteins during various pathological conditions, particularly those associated with disturbances in protein biosynthesis and with a marked change in the composition of serum proteins. The pathological conditions chosen were: protein deprivation, resulting in severe inhibition of liver protein and serum protein synthesis, liver damage caused by chloroform or carbon tetrachloride, and kidney damage caused by the introduction of heavy metals or by treatment with specific cytotoxic serum.

EXPERIMENTAL METHOD

Albino rats, weighing 120-150 g were used throughout. Electrophoretic and immunological tests with the tissue and serum proteins have been described in a previous communication [1]. Protein deficiency in animals was established by feeding them, over a period of two to six months, a diet evolved in this laboratory [2], containing only 3% protein (casein), but otherwise complete with respect to energy requirements, salt, and vitamin content, etc.

Liver damage was achieved by the daily administration of 1 ml of a 2% solution of CCl_4 in peach oil per 100 g body weight for one month, followed by 2 ml of a 10% solution three times a week. Animals receiving $CHCl_3$ were placed for 1-2 hours daily in a chamber containing a known concentration of chloroform vapor. Kidney damage was obtained by injection of uranyl nitrate or by the introduction of a cytotoxic serum obtained by the method of Mazug through immunization of rabbits with rat-kidney homogenates. The serum was introduced intravenously, 1 ml being given daily for three days, while there was no increase in urine protein, and the urine sediment did not show the presence of erythrocytes and casts, indicating the onset of kidney damage.

EXPERIMENTAL RESULTS

Electrophoretically demonstrable changes in liver and serum protein composition during protein starvation were similar to those already described in previous communications [3]. Some protein subfractions, corresponding

TABLE 1

The Content of Tissue Proteins Immunologically Identical With the Serum Proteins in Normal Animals and in Protein Deficiency (in percent of the total protein content of the given fraction)

Fraction corresponding to	Liver proteins		Kidney proteins		Brain proteins	
	normal	protein deficient	normal	protein deficient	normal	protein deficient
Serum albumins (IV)	93.0	53.7	100.0	75.0	36.0	40.0
Serum β-globulins (II)	2.7	2.8	5.7	0	3.9	4.3
Serum γ-globulins (I)	6.5	9.9	4.5	12.0	5.8	0

in mobilities to the serum α-globulins, disappeared completely, or their concentrations were greatly reduced. The mobilities of the proteins electrophoretically similar to serum albumins, β- and γ-globulins were not changed. A completely different picture was presented by the immunological properties of these proteins (Table 1).

Presented in Table 1 are the mean results of twelve experiments, the individual results in all experiments showing the same trend, and differing only in the degree of change.

As seen from the data presented, protein deficiency in the rat caused a fall (almost to half the normal value) in the amount of liver proteins immunologically identical with the serum albumins. No noticeable change occurred in the concentrations of proteins immunologically identical with the serum β-globulins (electrophoretic fraction II of the liver proteins). On the other hand, the content of proteins immunologically identical with the serum γ-globulins (electrophoretic fraction I of the liver proteins) increased considerably.

Distinctive changes in the immunological properties of the various kidney proteins were also observed. The content of proteins immunologically identical with the serum albumins fell by 25%, the proteins which corresponded immunologically to the serum β-globulins disappeared completely, while those corresponding to the serum γ-globulins increased in their content.

The changes observed in the brain during protein deficiency differed markedly from the corresponding changes in liver and kidney. In the brain, the amounts of proteins immunologically identical to the serum albumins remained unaltered during protein deficiency. The same applied to the proteins identical with the serum β-globulins, while the contents of protein fractions identical with the serum γ-globulins fell to zero level.

The data presented above were obtained with rats which were kept on a protein-deficient diet for 4-12 weeks, and in which the demonstrations of protein deficiency, particularly hypoproteinemia, were not very clear. A second group of rats was kept on the deficient diet for a period of 20-24 weeks, and the symptoms of protein deficiency in the animals of this group were quite clearly demonstrated; the results obtained with these animals are presented in Table 2.

TABLE 2

The Content of Tissue Proteins Immunologically Identical With the Serum Proteins During Severe Protein Deficiency in the Rat (in percent of the total protein content of the given fraction)

Fraction corresponding to	Liver proteins		Kidney proteins		Brain proteins	
	normal	protein deficient	normal	protein deficient	normal	protein deficient
Serum albumins (IV)	93.0	0	100.0	0	36.0	0
Serum β-globulins (II)	2.7	1.93	5.7	Traces	3.9	4.8
Serum γ-globulins (I)	6.5	11.5	4.5	13.6	5.8	5.3

It will be seen from Table 2 that in severe protein deficiency the changes in the content of proteins immunologically identical with the serum albumins were very sharply demonstrated. In all experiments these proteins were absent from nearly all tissues investigated. On the other hand, the changes in the contents of the

TABLE 3

The Content of Tissue Proteins Immunologically Identical With the Serum Proteins in the Rat Following Liver Damage Caused by CCl_4 and $CHCl_3$ (in % of total protein content of the given fraction)

Fraction corresponding to	Liver proteins			Kidney proteins			Brain proteins		
	normal	$CHCl_3$-poisoned	CCl_4-poisoned	normal	$CHCl_3$-poisoned	CCl_4-poisoned	normal	$CHCl_3$-poisoned	CCl_4-poisoned
Serum albumins (IV)	93.0	55.0	80.0	100.0	94.0	88.0	36.0	63.0	48.3
Serum β-globulins (II)	2.7	3.2	3.0	5.7	7.6	1.9	3.9	7.6	4.2
Serum γ-globulins (I)	6.5	1.7	6.5	4.5	11.5	3.1	5.8	22.0	4.7

protein fractions I and II, immunologically identical with the serum β- and γ-globulins, respectively, were of the same order as those observed in the case of protein deficiency of medium severity.

Presented in Table 3 are the results dealing with liver damage caused by administration of chloroform and of carbon tetrachloride.

It will be seen from this table that, in the liver, the content of proteins immunologically identical with the albumins fell both in the case of $CHCl_3$ and of CCl_4 poisoning, but that in the case of CCl_4 poisoning the fall in the concentration of the fraction identical with serum albumin was less pronounced than in the case of $CHCl_3$ poisoning, or of protein deficiency.

In the kidney the content of the protein fraction immunologically identical with the serum albumins fell only in the case of CCl_4 poisoning, and only slightly in the case of $CHCl_3$ poisoning, while in the brain their content increased under those conditions. The content of proteins immunologically identical with the β- and γ-globulins did not undergo any pronounced changes in any of the organs during $CHCl_3$ and CCl_4 poisoning, with the exception of a considerable increase in the brain and kidney of the proteins immunologically identical with the serum γ-globulin.

The data dealing with kidney damage have been presented in Table 4.

It will be seen from Table 4 that in the liver and kidney there was a considerable fall in the content of proteins immunologically identical with the serum albumin, the changes, both in liver and kidney, being more pronounced in the case of treatment with the specific cytotoxic serum than in the case of uranyl nitrate poisoning, even though the morphological changes in the kidney were more pronounced in the latter case. The content of proteins immunologically identical with the serum γ-globulins, increased markedly both in the liver and kidney in the uranyl nitrate injury. Kidney damage by the specific cytotoxic serum resulted, both in the liver and kidney, in a decreased content of the protein fractions immunologically identical with the serum γ-globulins, and in the liver alone – of those identical with the serum β-globulins.

The above results dealing with the effect of liver and kidney damage on the immunological properties of some liver proteins, particularly those identical by their electrophoretic mobilities and immunological properties with the serum albumins, suggested that the proteins of serum itself, whose albumins are synthesized in the liver, may also undergo some immunological changes under those conditions. This possibility was checked in a special experimental series. Antisera were prepared for a number of individual, electrophoretically homogeneous serum protein fractions, and with their aid, estimations were carried out, in animals in the various pathological states, of the amounts of proteins immunologically identical with the normal serum proteins.

Control experiments showed that the antisera prepared precipitated completely (100%) the corresponding protein fractions of the normal serum.

It will be seen from the data presented in Table 5 that during protein deficiency the entire serum albumin underwent a change in its immunological properties and that it became completely different from normal serum albumin. It is noteworthy that the electrophoretic mobility of

TABLE 4

The Content of Tissue Proteins Immunologically Identical With Serum Proteins in the Rat, Following Kidney Damage Induced by Uranyl Nitrate Poisoning or by the Introduction of Specific Cytotoxic Serum (in percent of the total protein content of the given protein fraction)

Fraction corresponding to	Liver proteins			Kidney proteins		
	normal	damaging agent		normal	damaging agent	
		cytotoxic serum	uranyl nitrate		cytotoxic serum	uranyl nitrate
Serum albumins (IV)	93.0	23.8	61.2	100.0	33.1	36.0
Serum β-globulins (II)	2.7	0.8	2.5	5.7	1.2	3.8
Serum γ-globulins (I)	6.5	2.4	8.8	4.5	5.6	15.5

TABLE 5

The Content of Serum Proteins Immunologically Identical With Normal Serum Proteins in Protein-Deficient Animals and in Animals Suffering from Liver Damage (in percent of the total protein content of the given fraction)

Serum fraction	Syndrome				
	protein starvation	$CHCl_3$ poisoning	CCl_4 poisoning	response to cytotoxic serum	uranyl nitrate poisoning
Albumins	0	98.0	87.5	96.8	48.0
β-globulins	22.7	81.6	95.8	27.2	64.0
γ-globulins	94.4	96.2	100.0	68.0	43.0

the albumin fraction remained unaltered during the deficiency. There were also marked changes in the immunological properties of the serum albumin during the uranyl-induced nephritis. Considerable changes in the immunological properties of the serum β-globulins were also observed during protein deficiency and following kidney damage. These changes were but faintly demonstrated in the case of liver damage following $CHCl_3$ and CCl_4 administration.

It should be noted that the γ-globulins, which are synthesized in the liver, did not undergo any change in their immunological properties during protein deficiency; however, during kidney damage, the amount of γ-globulins immunologically identical with the normal serum γ-globulins fell considerably.

DISCUSSION OF RESULTS

Previous investigations from this laboratory, conducted with the aid of Tiselius electrophoresis apparatus or with paper electrophoresis, allowed the conclusions that protein deficiency, as well as liver damage induced by treatment with CCl_4 or $CHCl_3$, brought about considerable changes in the quantitative interrelation between the various protein fractions of the liver, kidneys and serum.

The results presented in this communication indicate that, apart from these quantitative changes in the interrelation between the various fractions, there were also changes in the qualitative properties of the proteins which make up each fraction. These qualitative changes had no effect on the electrophoretic mobility of the various proteins, but they resulted in a radical change in their ability to react with corresponding specific antibodies. No reference to such changes in the tissue proteins could be found in the literature, while the published data dealing with the changes in the immunological properties of serum proteins suffers from considerable disagreement.

Thus Rother et al. [4], in the investigation of nephritis induced by the administration of cytotoxic serum,

reported clearly demonstrated changes in the immunological properties of the serum albumin. Similar results were obtained by Goettsch et al. [5] in their investigation of serum proteins in patients with nephrotic syndrome. In contrast to these results, Gitlin [6] found no difference in the immunological behavior between the serum albumin of children suffering from kidney damage, and that from normal children. Grabar et al. [7] also reported that the serum albumins obtained from human subjects with liver damage did not differ immunologically from the albumins obtained from healthy subjects. These discrepancies were probably due to the employment, by some investigators, of albumin preparations of various degree of purity for immunization purposes, which inevitably reflected in the results.

It has been the repeated experience of the present authors that the presence of even slight contaminations of other proteins in the albumin fraction used for immunization resulted in greatly distorted final results.

The discrepancy in data obtained from the study of patients with various types of liver and kidney damage may have been due to the different character of the individual syndrome. This is clearly borne out by the results obtained in the present study of kidney damage induced by the two different agents – cytotoxic serum and uranyl nitrate. While the introduction of uranyl nitrate resulted in heavier cytological damage, the more pronounced changes in the immunological properties of the liver proteins were caused by the cytotoxic serum.

All investigations were carried out with thoroughly purified, electrophoretically homogeneous proteins. The immunological differences were found not only in the albumin fraction, but also in other fractions of the serum proteins. The results prompt the conclusion that, in the pathological states covered by the present investigation, there took place in the animals, immunological changes in the proteins of the serum as well as of the various organs. It is at present impossible to state the nature of the changes which alter the immunological properties of these proteins without affecting their electrophoretic mobility. There is no doubt, however, that the reported changes in the immunological properties of serum and tissue proteins reflect certain shifts in the protein-synthetic mechanisms in the tissues, and that, in the first instance, these shifts bring about certain alterations in those centers of the protein molceules which are concerned with their immunological properties and specificity.

SUMMARY

Protein starvation in rats resulted in sharp changes of the immunological properties of liver, kidney, brain and serum proteins. The content of proteins immunologically identical with serum albumin fell considerably in liver and kidney. The proteins identical with the serum β-globulins disappeared completely from the kidney, while those identical with the serum γ-globulins disappeared from the brain.

Liver damage induced by CCl_4 or $CHCl_3$ also resulted in a fall, both in the liver and kidney, of the content of proteins immunologically identical with the serum albumins, while in the brain, under the same conditions, there was a definite increase in the content of the same fraction. During liver damage caused by chloroform the content of proteins, immunologically identical with the serum β-globulins, decreased in the kidney, but increased in the brain. The content of proteins immunologically identical with the serum γ-globulins fell in the liver, but increased in the kidney and brain.

Kidney damage caused by uranyl nitrate poisoning or by the introduction of cytotoxic serum caused an increase in the content of proteins immunologically identical with the serum albumins and β-globulins, both in the liver and kidney.

The immunological properties of the various protein fractions of the serum itself also underwent changes during the various pathological conditions studied, the changes in immunological properties of the serum albumins being particularly pronounced. There were no changes in the electrophoretic mobilities of the various tissue and serum proteins during the pathological conditions investigated.

LITERATURE CITED

[1] S.Ya. Kaplanskii, A.E. Gurvich and L.K. Starosel'tseva, Biokhimiya 23, 114 (1958).*

[2] S.Ya. Kaplanskii, N.N. Berezovskaya and Zh.G. Shmerling, Biokhimiya 10, 401 (1945).

*Original Russian pagination. See C.B. translation.

[3] S.Ya. Kaplanskii and O.B. Kuzovleva, Biokhimiya 22, 162 (1957).*

[4] R. Rother, H. Larre, R. Kluthe, E. Fischer and A. Schütze, Z. ges. exptl. Med. 129, 87 (1957).

[5] E. Goettsch and R. Reeves, J. Clin. Invest. 15, 173 (1936).

[6] D. Gitlin, Amer. J. Diseases Children 83, 82 (1952).

[7] P. Burtin, R. Fauvert and P. Grabar, Semaine hopitaux, Paris 2, 31 (1955).

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*Original Russian pagination. See C.B. translation.

THE USE OF PEPTIDES IN THE BIOSYNTHESIS OF PROTEINS

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The question of the nature of the intermediate products in the synthesis of macromolecules of protein has long been discussed in the biochemical literature. The widely accepted theory of the synthesis of proteins on a matrix of ribo- or desoxyribonucleic acid, which determines the order of succession of the amino acids in the polypeptide chain, assumes the single-act character of protein synthesis directly from amino acids which have first been arranged on the matrix surface in a definite order. However, data are accumulating which indicate that even in the presence of a matrix the synthesis can occur in a number of steps with the formation of intermediate products. Some very interesting studies to this effect by several recent authors [1-3] have shown that RNA from different sources, carefully freed from protein, contains firmly bound peptide fragments. The intermediate formation of peptides during active protein synthesis has recently been shown in a series of studies with microorganisms [4-7] and with plant and animal subjects [8-10]. At the same time there is material in the literature on the formation of proteins from a reserve stock of amino acids [11-13], but in most cases these materials are of an indirect nature and do not show the mechanism of the synthetic reaction.

The question of the direct utilization of peptides and polypeptides in protein synthesis and the specificity of their utilization takes on great significance in connection with the interesting study of Ebert [14] who found that when some chick organ was transferred to the chorion allantois of the chicken embryo, the products of incomplete splitting of the transplant proteins were selectively used for the synthesis of proteins of the homologous organ.

In view of all this work, it has seemed to us that the peptide fraction of organs and tissues has attracted much more attention in recent times than in previous years. Starting from the idea of the importance of the role of peptides in carrying out the synthesis of proteins and producing their specific structures [15], we began a series of studies on the metabolism of peptide products in plant leaves. In the first study [16] we tried to establish the main possibilities of the direct use, by plants, of peptide products split from the corresponding plant proteins. In the present study we consider it expedient, in view of Ebert's work, to follow also the possibility of selective use of peptides, but not in the species or organ-tissue aspect, rather with respect to the synthesis of proteins in different intracellular fractions of the peptide products from the splitting of proteins of the same fractions.

METHODS

Principle of the method. The experiment was run as follows. From a plant leaf kept in a chamber with an atmosphere of $C^{14}O_2$ we separated the plastid proteins and the rest of the cytoplasm, which after removal of the cell sap, plastids, and nucleus, was composed chiefly of cytoplasmic granules. The isolated proteins labeled with C^{14} were submitted to partial hydrolysis by pepsin (to peptides with an average particle diameter of 5.5 amino acid residues) and to full hydrolysis to amino acids with 6 N HCl. The resulting hydrolysates, after they had been freed from unhydrolyzed protein and HCl and with neutralization, were introduced into the leaves of a new group of plants with later determination of the uptake of activity in the proteins of the same fraction and with calculation of the activity evolved in the form of carbon dioxide.

The results of such determinations allowed us to judge the direct character of the use of peptides in the synthesis of proteins. When labeled C^{14} was introduced into the leaf as fully hydrolyzed protein and it was kept

in the dark, only part of the activity of the amino acids was included in the protein. Another considerable part of the added amino acids, because of the absence of photosynthesis (exposure in the dark), underwent oxidative decomposition with evolution of $C^{14}O_2$. If we assume that the utilization of peptides in protein synthesis requires their preliminary splitting into amino acids, then when the same amount of C^{14} is added to the proteins in both variants, the active carbon dioxide evolved should also be similar in amount. If labeled peptides are taken up, the protein has the same or greater activity than when free amino acids are added; the activity evolved in oxidation should be considerably less, which would be the result of direct utilization of peptide fragments in the synthesis of proteins.

In previous work [16] this finding was made in comparing the use of protein fully hydrolyzed or hydrolyzed by pancreatic enzymes. The pancreatic hydrolysate consisted of comparatively small peptides (average diameter 2.8-3.2 amino acid residues). In the present study we have used a mixture of larger peptide fragments, and therefore the comparison of the results of both studies in this respect can also have a definite interest.

These experiments were carried out in the autumn and winter of 1957-1958, and the plants for the experiments were grown under artificial illumination with a daily illumination by a luminescent lamp. To obtain the labeled protein, we picked about 50 month-old bean plants at the main stems and submerged them at the cut in water, in test tubes which were placed on a support in a hermetically sealed glass chamber with a volume of 0.2 m^3 (length 100 cm, width 40 cm, height 50 cm). The first layer of leaves and the leaflets in the upper portion were removed before beginning the exposure, so that for the assimilation of $C^{14}O_2$ there remained only the well-developed leaves of the second to the fourth layer. We introduced 2 mC of $C^{14}O_2$ into the chamber and over a period of 48 hours (with two eight-hour night intervals) the chamber was illuminated with a daylight lamp. During this time practically all the carbon dioxide underwent biochemical transformation, as a result of which, according to preliminary calculations based on the radioactivity of the proteins, these had concentrated about 125-150 μC in their composition. We isolated the plastid and the cytoplasm proteins separately from the leaves (300 g). The plastids were separated by centrifuging at 3600 g for 30 minutes at low temperature. Because C^{14} was present in most of the organic substances of the leaves, the purification of the proteins with C^{14} was carried out with particular care. Beside the usual methods of reprecipitating and defatting with alcohol and ether, we also used, for the purification of the proteins labeled with radiocarbon, the procedures of washing the protein with hot 5% trichloroacetic acid (TCA) (to remove nucleic acids), and treating with amylase (to hydrolyze possible admixtures of starch).

The radioactivity of the plastic proteins isolated in this way was 43,870 counts/min per 10 mg, and of the cytoplasm proteins, 47,870 counts/min per 10 mg.

Hydrolysis and proteolysis of the proteins in all the experiments were carried out by the same procedures. Complete hydrolysis of the proteins was attained by boiling them with 20% HCl for 20 hours under reflux. The hydrolysate was freed from HCl by repeated evaporations.

Proteolysis of the proteins was carried out with crystalline pepsin. The resulting pepsin hydrolysate was neutralized with a solution of NaOH, heated to inactivate the enzyme, and the unhydrolyzed portion of the protein was precipitated by adding TCA to the hot solution. Plant proteins are usually not completely split by pepsin, and the product always contains some portion which is precipitated by TCA. After the precipitate was centrifuged off and the solution filtered, the pepsin hydrolysate of the protein was freed from TCA by repeated extraction with ether in a separatory funnel (to a neutral reaction) and the ether was evaporated on the water bath.

The hydrolysates used for comparison in each series of experiments were brought to the same radioactivity by diluting them with a 0.1 N solution of NaCl. The complete hydrolysates were also brought to the activity of the pepsin hydrolysates and to the same NaCl content. All the working solutions were equalized as to pH by adding to them a phosphate buffer with pH 6.8. Before using the hydrochloric acid hydrolysates, we added to them an amount of autolyzed pepsin which corresponded to that in the pepsin hydrolysates. Thus, the solutions of hydrolysates used for comparison in each series of experiments were equalized as to concentration, pH, and radioactivity. Tryptophan (1%) was also added to the complete hydrolysate to replace that destroyed in the hydrolysis. After the hydrolysates had been prepared, they were sterilized and kept under sterile conditions in the refrigerator.

Analysis of the amino acids in the pepsin hydrolysate by the Van Slyke method, before and after complete hydrolysis, showed that the average diameter of the peptides in this hydrolysate varied from 5.2 to 5.6 amino acid residues in different experiments.

TABLE 1

The Use of Different Hydrolysates from Bean Proteins Labeled With C^{14} in the Synthesis of Plastid and Cytoplasm Proteins and in Oxidative Processes in Bean Leaves*

Nature of protein hydrolysate labeled with C^{14} introduced into plant	Plastid protein			Cytoplasm protein			$BaCO_3$ precipitate			Ratio of total activity of protein and total activity of $BaCO_3$	Total activity of nonprotein C^{14} fraction
	weight of protein in sample (mg)	specific activity, in counts per min per 10 mg	total activity, in counts per min	weight in sample (mg)	specific activity, in counts per min per 10 mg	total activity, in counts per min	weight of precipitate in sample (mg)	specific activity, in counts per min per 10 mg	total activity, in counts per min		
1st Experiment (introducing hydrolysates with activity 7000 counts/min per ml)											
Complete hydrolysate											
after 6 hours	124	57	706.8	239	56	1338	460	71	3266	0.62	4300
after 12 hours	173	89	1539	242	115	2760	1217	83	10126	0.42	8950
Pepsin hydrolysate of plastid proteins											
after 6 hours	128	102	1305	230	100	2300	585	55	2942	1.33	4550
after 12 hours	132	114	1482	269	149	4023	1139	40	4560	1.21	7900
Pepsin hydrolysate of cytoplasm proteins											
after 6 hours	157	83	1303	293	113	3277	437	67	3263	1.40	4190
after 12 hours	142	102	1448	277	152	4250	1061	51	5406	1.05	8150
2nd Experiment (introducing hydrolysates with activity 15,400 counts/min per ml)											
Complete hydrolysate of proteins after 12 hours	80	96	768	130	147	1911	1322	59	7788	0.34	14800
Pepsin hydrolysate of cytoplasm protein after 12 hours	70	234	1638	132	405	5270	880	110	9680	0.71	19250

*The variation in weight of protein is chiefly due to small differences in wet weight of the plants in the different chambers.

TABLE 2

The Utilization of Different Hydrolysates from Labeled Proteins by Bean Leaves Under Different Experimental Conditions

Nature of hydrolysate from proteins labeled with C^{14} introduced into the plant	Total protein			$BaCO_3$ precipitate			Ratio of total protein activity to total $BaCO_3$ activity	Total activity of nonprotein fraction
	weight of protein in sample (mg)	specific activity, in counts/min per 10 mg	total activity, in counts/min	weight in sample (mg)	specific activity, in counts/min per 10 mg	total activity, in counts/min		
Effect of concentration								
Exposure 12 hours								
Complete hydrolysate								
Activity 18,000 counts/min per ml	175	183	3111	590	180	10620	0.29	13000
Activity 6000 counts/min per ml	197	49	965	675	63	4252	0.23	4500
Pepsin hydrolysate								
Activity 18,000 counts/min per ml	212	452	9492	741	156	11544	0.82	2150
Activity 6000 counts/min per ml	170	150	2550	660	61	4026	0.64	8500
Exposure 3 hours								
Complete hydrolysate								
Activity 18,000 counts/min per ml	185	48	888	320	60	1920	0.46	9500
Pepsin hydrolysate								
Activity 18,000 counts/min per ml	210	79	1659	330	48	1584	1.05	14500
Effect of glucose on oxidation of C^{14} products								
Introduced hydrolysate with activity of 20,000 counts/min per ml								
Complete hydrolysate								
a) without glucose	313	163	5053	581	256	14848	0.34	28800
b) with addition of 2% glucose	270	120	3240	608	230	13984	0.23	26400
Pepsin hydrolysate								
a) without glucose	238	321	7616	573	145	8265	0.91	34600
b) with addition of 2% glucose	300	283	8490	715	124	8801	0.96	35200

Comparison of metabolism of incomplete acid and pepsin hydrolysates introduced hydrolysate with activity of 20,000 counts/min per ml

Nature of hydrolysate from proteins labeled with C^{14} introduced into the plant	Total protein			$BaCO_3$ precipitate			Ratio of total protein activity to total $BaCO_3$ activity	Total activity of nonprotein fraction
	weight of protein in sample (mg)	specific activity, in counts/min per 10 mg	total activity, in counts/min	weight in sample (mg)	specific activity, in counts/min per 10 mg	total activity, in counts/min		
Complete hydrolysate	294	178	5162	625	275	17050	0.30	21000
Incomplete acid hydrolysis (av. diameter of peptides 2.5 amino acid residues)	235	240	5640	680	212	14416	0.39	23500
Pepsin hydrolysate (av. diameter of peptides 5.4 amino acid residues)	267	248	6696	662	110	7882	0.91	21500

Introduction of the hydrolysates into the leaves and calculation of the uptake of radioactivity in the proteins. In the experiments with hydrolysates of proteins labeled with C^{14} we used for their introduction into the leaves both the transpiration current and the phloem as a result of immersing the cut stem (in the portion above the cotyledons) in a tube with the corresponding hydrolysate. The uptake of radioactivity in the leaf proteins and the giving off of labeled carbon dioxide was observed after 50-60 minutes from the immersion. In typical experiments several plants (wet weight of leaves from 10 to 15 g) placed in a hermetically sealed glass chamber with volume 10 liters, in the dark at 20°, absorbed labeled hydrolysate through the cut stem for 3-12 hours. The carbon dioxide and water given off by the leaves were absorbed by dry alkali (25 g in each chamber). Regeneration of the oxygen absorbed by the leaves took place by the evolution of oxygen from hydrogen peroxide in a small beaker placed in the chamber. The radioactivity was determined in the proteins of the different fractions separated from the leaves of the experimental plants and also in the precipitate of $BaCO_3$ obtained after addition of $BaCl_2$ to the solution of the alkali placed in the chamber. Measurement of the activity was carried out in the precipitate from a thick layer spread over a surface of 4.5 cm^2 with later calculation per 10 mg from the experimentally established curve of autoabsorption of preparations of equal weight. The details of this measurement have been described earlier by us [16-18]. For all the measurements we used a T-BFL-25 counter with a thin mica window (1 mg/cm^2).

Reproducibility. In most cases for each experiment we compared the results of introducing three hydrolysates. In view of the complex preparative work with the plants, we could not carry out more than two repeated experiments. The figures in the table are the result of the average of two repetitions. However, the basic principle of the scheme was the same in all experiments which increases the reliability of the final conclusions which are made on the basis of the total results of all the experiments.

RESULTS

In the first experiments with protein hydrolysates with random labeling by C^{14}, we used, for the introduction into plants, hydrolysates which were first equalized to an activity of 7000 counts/min per ml. The total activity for the liquid samples was calculated in the same way as for the solid, that is, by calculating over the surface activity to 10 mg and then calculating on some weighed amount of liquid. In the leaves used for analysis in the first and later experiments, the radioactivity was determined not only in the proteins, but also in the nonprotein fractions so as to calculate the total amount of hydrolysate which entered the leaves. We thus observed that the pepsin hydrolysate of proteins was not only used more fully in protein

synthesis, but in some cases was absorbed more rapidly from solution by the plant. The data of the first experiment are given in Table 1. In the same table we also give the material from the second experiment, in which we introduced hydrolysates with higher concentrations of amino acids and peptides (15,400 counts/min per ml) into the plant.

Analysis of the data of the table, with respect to the specific activity of the proteins, clearly shows the absence of any selective uptake of peptides in the separate intracellular fractions, and the products of incomplete hydrolysis of the plastid proteins are used, with the same success, for uptake by cytoplasm proteins and vice versa. The cytoplasm in all cases took up the isotope with greater speed than did the plastids.

From an inspection of the figures in the next to the last column of the table, it is not difficult to establish that peptides, considerably more actively used in protein synthesis than are the free amino acids of the complete hydrolysate, are 2-3 times less active in oxidative transformations which occur with evolution of labeled carbon dioxide. This indicates that only part of the compounds of this fraction is split to amino acids; the greater part is used directly in the synthesis of protein. This ratio between the amount of peptide used in protein synthesis and in oxidative reactions is shown for the larger peptides of the pepsin hydrolysis of proteins to almost the same degree as for the pancreatic hydrolysate which we studied earlier [16].

Since the hydrolysates enter the plant in proportion to the wet mass, the small differences in weight of the plants in the samples cannot be reflected in the specific activity of the proteins and the corresponding total activity of proteins and $BaCO_3$.

From the standpoint of energetics, the more rapid uptake of radioactivity in proteins from the peptide products which correspond to the structure of the formed protein is quite understandable; a considerably smaller number of peptide bonds must be formed in this case for the formation of the same amount of protein. A control variant, keeping the hydrolysate in the chamber without a plant, showed that in the first six hours there was no evolution of labeled carbon dioxide from the surface of the amino acid or peptide solutions. After 12-hour exposure, a small amount of $C^{14}O_2$ was evolved; in this case the $BaCO_3$ precipitate had a slight activity, of about 200-250 counts/min in the whole mass of the precipitate, that is, about 1/50-1/25 the total activity of the precipitate in the experimental variants. The formation of this carbon dioxide was evidently related to the initial development at this time of hydrolyzing microflora. A second control, set up to test the scale of nonspecific adsorption of labeled amino acids and peptides by the protein, was designed to explain whether there existed any difference in activity of the protein because of stronger adsorption of the peptide. The fact of strong adsorption of labeled amino acids by proteins was established by a number of authors [19-21]. The addition of corresponding amounts of labeled hydrolysates to bean-leaf homogenates, killed by freezing showed that in spite of reprecipitation and careful washing, the proteins of plastids and cytoplasms adsorb some amount of radioactive products and acquire an activity of the order of 6-8 counts/min per 10 mg, independent of the nature of the hydrolysate. Evidently, therefore, since only about 6-10% of the activity of the protein is connected with adsorption, the differences between the variants actually depend on metabolic processes.

In the next series of experiments we did not separate the protoplasm into plastid and cytoplasm fractions. The determination of radioactivity in this case was carried out on the total preparation of proteins extracted from the leaves by 0.5% NaOH. The problem in these experiments was to explain the effect of the following three factors on the ratio of distribution of C^{14} activity between proteins and evolved carbon dioxide: concentration of hydrolysate, presence in the hydrolysate of carbohydrate substrates for leaf respiration, and method of obtaining the peptides (comparison between incomplete acid hydrolysates and enzymatic hydrolysates). In the last case, we wished to confirm the results of one of the experiments of our previous study [16] which showed that peptides obtained by incomplete acid hydrolysis of proteins differed in the character of their metabolism from those from enzymatic hydrolysis. The data of these experiments are given in Table 2.

The material in this table shows that neither the concentration of the hydrolysate nor the presence of glucose changes, in principle, the relation of total activity of protein to total activity of the carbon dioxide evolved from the plant during oxidation. In all cases assimilation of the peptides is characterized by a much greater uptake of activity in the protein as compared to its use for oxidation. At the same time, the peptides of the incomplete acid hydrolysate hardly differ in this respect from free amino acids. This result, which confirms our earlier data, shows that peptides obtained in different ways from splitting of the same protein are characterized by a differene metabolism.

SUMMARY

From a consideration of the results of this work,we can conclude that the data more or less reliably indicate only that peptides from the pepsin hydrolysis of proteins, introduced into plants, in most cases are used in the synthesis of proteins of the same plant more actively than are free amino acids. However, the splitting of peptides and the use of their amino acids in oxidative reactions is much less than with complete hydrolysates, which also indicates their direct use in synthetic reactions. The ability of the synthesizing system of plants to use existing peptides is quite understandable, especially if we consider that aside from the processes of local renewal of proteins, plants have a very widely distributed set of processes for transfer of the products of splitting proteins from one organ to another (in germination, autumn maturation, successive falling of different leaves, etc.). Raacke [9] has recently published some very interesting material in this field. When he studied the dynamics of amino acids, peptides, and proteins in the sprouting of pea seeds, he found that the pericarp first grew intensively and the proteins in it underwent a breakdown and a transformation to forming the cotyledons. He showed clearly that this splitting of proteins in the pericarp did not lead to free amino acids, and all the transfer of nitrogenous products to the cotyledons took place in the form of peptides and polypeptides, from which the seeds reconstructed new protein. Such an incomplete cycle of splitting and resynthesis is evidently more economical, since in the reconstruction of proteins in the cotyledons less energy is expended than in the synthesis from free amino acids.

In our experiments we do not find any particular selectivity in the uptake of peptides corresponding to the fraction of the original labeled protein from which they were isolated. This result may be related to a number of factors: first, to the presence of a considerable assortment of proteins with similar structures in the different fractions; second, with the presence of peptide groupings which are repeated in different combinations in different proteins [22]; and third, with sufficiently prolonged exposures in the experiments. There is no doubt that it would be expedient to carry out more study of the effects of specific syntheses in cell fractions and in organs and species of plants. Success of such investigations could offer a number of possibilities for selective stimulation of the growth of different tissues and organs.

LITERATURE CITED

[1] B. Keil' and M. Gruboshova, Coll. Czech. Chem. Works 20, 713 (1955).

[2] V. Koningsberger, Chr.O. Van Der Grinten and J.Th.G. Overbeek, Biochim. et biophys. acta 26, 483 (1957).

[3] S.L. Potter and A.L. Dounce, J. Amer. Chem. Soc. 78, 3078 (1956).

[4] G.E. Cohnell and R.W. Watson, Biochim. et biophys. acta 24, 226 (1957).

[5] S.J. Dagley and A.R. Johnson, Biochim. et biophys. acta 21, 270 (1956).

[6] A.G. Morton and D. Broadbent, J. Gen. Microbiol. 12, 248 (1956).

[7] F. Turba and H. Esser, Biochem. Z. 327, 93 (1955).

[8] N.M. Sisakyan and E.B. Kuvaeva, Biokhimiya 22, 639 (1957).*

[9] I.D. Raacke, Biochem. J. 66, 101, 110, 113 (1957).

[10] L.K. Ramachandran and T. Winnick, Biochim. et biophys. acta 23, 533 (1957).

[11] P.N. Campbell and N.E. Stone, Biochem. J. 66, 669 (1957).

[12] R. Britten, R.B. Roberts and E.F. French, Proc. Nat. Acad. Sci. USA 41, 863 (1955).

[13] S. Spiegelman, H.O. Halvorson and R. Ben-Ischai, Amino Acid Metabolism (Hopkins Press, Inc., Baltimore, 1955) p. 124.

[14] J.D. Ebert, Proc. Nat. Acad. Sci. USA 40, 159 (1955).

[15] Zh.A. Medvedev, Uspekhi Sovrem. Biologii 40, 159 (1955).

[16] Zh.A. Medvedev, Biokhimiya 21, 288 (1956).*

*Original Russian pagination. See C.B. translation.

[17] Zh.A. Medvedev and E.A. Fedorov, Fiziol. Rastenii 3, 547 (1956).

[18] Zh.A. Medvedev, Biokhimiya 22, 855 (1957).*

[19] A.G. Pasynskii, M.S. Volkova and V.P. Blokhina, Doklady Akad. Nauk SSSR 101, 723 (1955).

[20] S.E. Shnol', Vopr. Med. Khimii 1, 327 (1955).

[21] Zh.A. Medvedev, Doklady Akad. Nauk SSSR 117, 860 (1957).*

[22] F. Shorm, Coll.: The Origin of Life on the Earth [in Russian] (Izd. AN SSSR, Moscow, 1957) p. 166.

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COMPARATIVE METABOLIC CHARACTERISTICS IN CULTURES OF NORMAL AND CANCER CELLS

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In a previous communication we showed [1] that the growth of monkey kidney cortex cells under tissue culture conditions produces some changes in metabolism. After several days of culturing in vitro the normal cells acquire properties which are usually considered characteristic of cancer tissues.

Continuing this study, we have compared metabolism in cells of cultured normal tissue (seven- and ten-day cultures of kidney cortex of the monkey Macacus rhesus) and cells from the culture of cancer cells, type HEP-2 which originated from a malignant degeneration of human laryngeal epithelium [2]. At the same time, as a third object of study we used a strain of heart cells from the monkey Macacus cynomolgus (MH) whose nature is still not sufficiently clear [3, 4]. This epithelium-like strain has evidently been produced from normal cells which, during long culturing, have undergone a definite transformation. Interest was aroused in this strain because it has been widely distributed in virusological laboratories. The results obtained in studying metabolism in these three cultures furnish the subject of this communication.

EXPERIMENTAL METHOD

Culturing and preparing the cells. The methods used for trypsin treatment and tissue culturing were described in the previous communication. In some experiments trypsin treatment of the monkey kidney cortex was carried out by treatment of the tissue with trypsin in the cold [5]. The nutritive medium in all three types of culture was not changed. The cells of the HEP-2 and MH strains were grown on medium 199 [6] which contained 10% calf serum. Removal of the cells from the glass surface for reinoculation or for experiment was carried out after 7-10 days. We used a 0.02% solution of sodium ethylenediamine tetraacetate (EDTA) in a Dulbecco buffer for this [7]. The cells were washed in the same way as in the previous work, but instead of a solution of sodium chloride we used a Krebs-Ringer solution. For counting the cells we used the method of Sanford et al. [8] with some simplification, which consisted in disintegrating the cytoplasm with 0.1 M citric acid and counting the nuclei stained with crystal violet in a hemocytometer. We estimated the functional state of the cells by staining them with erythrosine B, which under definite conditions stains only damaged and obviously not metabolizing cells [9].

Biochemical methods. Manometric experiments and determination of glucose, lactic acid and the phosphorus of desoxyribonucleic acid (DNA), and also measurement of the rate of the succinic dehydrogenase and hexokinase reactions were carried out as in the previous work. Destruction of the cells for study of enzyme activity was carried out by freezing and thawing three times. Since sometimes, especially in work with the HEP-2 strain, we found intense endogenous reduction of neotetrazole (that is, without addition of succinate), we carried out a series of experiments with a washed homogenate. The washing was performed by centrifuging three times at about 20,000 g for ten minutes, with resuspension in a $5 \cdot 10^{-4}$ M EDTA solution. Special tests showed that under the conditions of our experiments the rate of the studied reaction was proportional to the amount of enzyme. All the metabolic properties were converted to a base of 10^6 cells.

EXPERIMENTAL RESULTS

Content of DNA phosphorus in the cell nuclei. Beside the fact that the content of DNA phosphorus in the cell nuclei is of intrinsic interest, its determination is important for us for the comparison of our data with the results of the previous study, in which all the metabolic coefficients were calculated per 1 μg P of DNA. The results of the determination are given in Table 1. The values obtained for the monkey kidney tissue cultures are very close to the value of 0.83 μμg P of DNA per nucleus obtained by other authors for human kidney cells not cultured in vitro [10]. Evidently, this value corresponds to the normal diploid condition of the chromosomes. The nuclei of the HEP-2 cancer cells contain a definitely increased amount of DNA P, which agrees with the literature data [11] on the heteroploid condition of the chromosomes in such cells. It should be mentioned that, according to our data, the HEP-2 cells contain somewhat less DNA phosphorus than according to the data from the laboratory of Leslie [12]. As Table 1 shows, the content of DNA phosphorus in the nuclei of MH cells also corresponds to a heteroploid condition of the chromosomes.

TABLE 1

Content of DNA Phosphorus in Cells of Tissue Cultures

Name of culture	DNA phosphorus in μμg per 1 nucleus
Monkey kidney culture	0.80 ± 0.03 (5)*
HEP-2	1.26 ± 0.04 (7)
MH	1.18 ± 0.02 (5)

*In the parentheses in this and later tables we give the number of experiments carried out.

Endogenous respiration. Since we wished to compare the metabolism in cultures of cancer cells with metabolism in cultures of normal cells, we considered it necessary to repeat our experiments with cultures of monkey kidney tissue, since some experimental conditions were changed (no change in medium, removal of cells from the glass surface with EDTA, etc.).

It should be noted that the intensity of respiration depends in great degree on the quantity of cells in the Warburg apparatus. When more liquid suspensions are used, the oxygen use is greater than when converted to 10^6 cells. Since in experiments with very viscous suspensions the total use of oxygen is several ml and cannot be determined with sufficient accuracy, we worked with suspensions of medium thickness and so our results are known to be somewhat low. However, by working with suspensions of the same thickness we could reproduce our results well enough and so they were suitable for comparisons.

When we compare the intensity of use of oxygen in samples without exogenous substrate (Table 2) we see that respiration in HEP-2 cancer cells is somewhat lower than respiration in cells from monkey kidney cultures. The intensity of use of oxygen by MH cells evidently does not differ from that of monkey kidney explant cells.

TABLE 2

Endogenous Respiration of Tissue Culture Cells (gas medium – air, t 37°, period of incubation 1 hour. Incubation mixture – Krebs-Ringer phosphate buffer, pH 7.3, 1.9 ml; 0.9% NaCl, 0.5 ml; cell suspension, 0.5 ml)

Name of culture	Average number of nuclei in sample	Average % of cells stained by erythrosine B	Oxygen requirement in μl per 10^6 nuclei
Monkey kidney culture	$21.6 \cdot 10^6$	35	3.3 (7)
HEP-2	$25.9 \cdot 10^6$	22	2.1 (7)
MH	$30.0 \cdot 10^6$	14	3.4 (8)

Respiration in the presence of succinate and p-phenylenediamine. The addition of succinate and p-phenylenediamine increases the use of oxygen by the cells studied relatively weakly (Table 3). In the presence of these substrates, respiration is increased by 16-99%. In this respect, the cells of the tissue cultures differ considerably from normal cells which have not been cultured in vitro, whose oxygen requirement in the presence of succinate and p-phenylenediamine is usually many times greater.

The weak response to the addition of p-phenylenediamine depends on the destruction in the cytochrome

TABLE 3

Effect of Different Substrates on Respiration of Tissue Culture Cells (conditions of experiment, see Table 2. Instead of the corresponding volume of 0.9% NaCl we added 0.2 M succinate, 0.1 ml; 4% p-phenylenediamine, 0.1 ml; 1.45% glucose, 0.2 ml)

Name of culture	% Change in respiration		
	in presence of succinate	in presence of p-phenylenediamine	in presence of glucose
Monkey kidney culture	+65 (9)	+99 (8)	−35 (10)
HEP-2	+23 (7)	+99 (7)	−24 (7)
MH	+16 (5)	+61 (5)	−31 (10)

portion of the catalysts for the respiratory chain. Thus, this destruction occurs both in cancer cells and in normal cells cultured in vitro. The weak ability of succinate to stimulate the oxygen requirement can depend on destruction of the respiratory cycle in the cytochrome portion and also on injury to the succinic dehydrogenase step in this cycle. To determine the correctness of this assumption, we carried out experiments on the rate of the succinic dehydrogenase reaction.

Rate of the succinic dehydrogenase reaction. The results obtained in comparative determination of the rate of the succinic dehydrogenase reaction in the cells of different tissue cultures (Table 4) indicate that the activity of this enzyme in all the tissue cultures studied was at a comparatively low level. For the sake of comparison we can say that in monkey kidney cortex tissue which has not been cultured in vitro the rate of the succinic dehydrogenase reaction corresponds to the formation of 11.1 μg of neobiformazane per 1 μg of DNA phosphorus or 13.9 μg of neobiformazane per 10^6 cells in 40 minutes [1]. Thus, in normal and cancerous tissue culture cells there occurs not only destruction of the cytochrome portion of the respiratory chain, but also a definite weakening of the succinic dehydrogenase portion.

TABLE 4

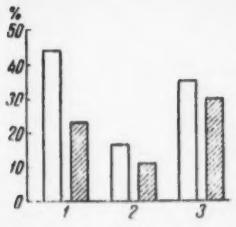
Rate of Succinic Dehydrogenase Reaction in Tissue Culture Cells (gas medium − air; t 37°; length of incubation 40 min. Incubation mixture: 0.1 M phosphate buffer, pH 7.4 − 1.0 ml, 0.2 M Na succinate − 0.5 ml, 0.5% neotetrazole chloride − 0.2 ml, 0.2% KCN − 0.1 ml, 0.9% NaCl − 0.2 ml, homogenate − 1 ml. In the control samples instead of succinate we placed a corresponding amount of 0.9% NaCl)

Name of culture	Formation of neobiformazane in μg per 10^6 nuclei
Monkey kidney culture	3.1 (8)
HEP-2	3.0 (3)
MH	4.8 (13)

Inverse Pasteur effect. We showed before that in normal cells from monkey kidney tissue after short culturing in vitro there was an inverse Pasteur reaction (Crabtree effect); the addition of glucose decreased the oxygen requirement of these cells. Glucose has exactly the same action on respiration in the other cultures studied (Table 3). Thus, the inverse Pasteur effect is inherent in normal and cancer tissue culture cells.

Although in our work we have not tried to explain the mechanism of the inverse Pasteur effect, yet in the course of other experiments we have observed the resistance of this reaction to monoiodoacetate (Figure). In the presence of such concentrations of iodoacetate as would almost completely suppress the formation of lactic acid, the addition of glucose still had a definite suppressing action on respiration. When some authors studied the inverse Pasteur effect in cells of an ascitic cancer, they also found that this effect was maintained even in the presence of monoiodoacetate [13, 14]. However, others have declared that the hindering of glycolysis in ascitic carcinoma practically fully restores the demand for oxygen in the glucose samples [15].

Glycolysis. The intensity of glycolysis (like the intensity of respiration) depends considerably on the number of cells in the Warburg apparatus. Thus, in five experiments on cells of monkey kidney tissue cultures with an average of $7.9 \cdot 10^6$ cells in the vessel, we obtained an average value for aerobic glycolysis of 56 μg of lactic acid per 10^6 cells, and in analogous experiments, but with an average content of $25.2 \cdot 10^6$ cells in the vessel, aerobic glycolysis gave only 21.7 μg of lactic acid per 10^6 cells. Therefore, the stipulation made as to the absolute value of the respiratory coefficient applies also to the absolute value of the glycolysis coefficient.



Effect of monoiodoacetate on the inverse Pasteur effect. On the ordinate – % inverse Pasteur effect; white columns – without monoiodoacetate; cross-hatched columns – in the presence of $5 \cdot 10^{-4}$ M monoiodoacetate: 1) monkey kidney culture (suppression of glycolysis in the presence of monoiodoacetate reaches 93%); 2) HEP-2 (suppression of glycolysis, 93%); 3) MH (suppression of glycolysis, 97%).

The results are given in Table 5. The cells of all three cultures studied have very high aerobic and anaerobic glycolysis. It is interesting that in the HEP-2 cancer cells, under the conditions of our experiments, glycolysis is evidently even lower than in the normal cells of the monkey kidney culture (judging by the decrease of glucose). This finding agrees with the data of Leslie and co-workers that aerobic glycolysis in cells of the cancer strains are usually lower than in cells of explants of normal embryonic tissue [12]. As Table 5 shows, cells of the MH strain occupy, as it were, an intermediate position between the HEP-2 cells and the monkey kidney culture cells in intensity of glycolysis.

The data of this table indicate that in the HEP-2 cells practically all the glucose which disappears under the conditions of our experiments goes to lactic acid. Differing from this, in the other investigations, a considerable part of the glucose was utilized and not transformed into lactate.

Rate of hexokinase reaction. In the previous communication we showed that the rate of the hexokinase reaction in kidney cultures was considerably higher than the rate of this reaction in starting cells which were not cultured in vitro. Comparison of the rate of the hexokinase reaction in cells of the different tissue cultures indicates that the hexokinase activity in these cells is relatively high (Table 6). For cells of monkey kidney tissue we obtained a somewhat lower value than in the previous work [1]. Most probably this is related to methodological conditions (different ATP preparation, different method of breaking up the cells, etc.). Special confirmatory experiments carried out with the starting trypsin-treated but not cultured in vitro cells of monkey kidney cortex showed that in these cells the rate of the hexokinase reaction was less than in the experiments of the last year (the fall in glucose was 0.8 μg of glucose per 10^6 cells in eight minutes). On such a low base the increase in hexokinase activity in the tissue culture cells was still more evident.

It is interesting that when we compare the rate of the hexokinase reaction in the different tissue cultures, we have the same picture as when we compare the glycolytic activity: the normal cells in the tissue cultures have the greatest hexokinase activity, the HEP-2 cells, the least, and the MH cells occupy an intermediate position.

TABLE 5

Glycolysis in Cells of the Tissue Cultures (experimental conditions, see Table 2. In the anaerobic experiments the gas phase was nitrogen. Instead of the corresponding volume of 0.9% NaCl, we added 0.2 ml of 1.45% glucose)

Name of culture	Average No. of nuclei in sample	Average % of cells stained by erythrosine B	Fall in glucose in μg/10^6 nuclei		Accumulation of lactate in μg/10^6 nuclei	
			aerobic	anaerobic	aerobic	anaerobic
Monkey kidney culture	$25.2 \cdot 10^6$	30	41.3 (4)	38.8 (4)	21.7 (5)	23.9 (5)
HEP-2	$30.7 \cdot 10^6$	21	18.8 (4)	18.4 (4)	15.1 (5)	20.0 (5)
MH	$29.0 \cdot 10^6$	14	28.0 (5)	34.0 (5)	17.2 (5)	20.0 (5)

DISCUSSION OF RESULTS

Specificity of cancer metabolism. In our work we noticed that in a number of important respects the metabolism in the cancer cell cultures resembled the metabolism in normal cell cultures.

The basic idea of the special point of cancer metabolism was expressed by Warburg 30 years ago [16].

TABLE 6

Rate of the Hexokinase Reaction (gas medium – air, t 30°, length of incubation 8 min. Final concentration: Na phosphate buffer, pH 7.8 – $4 \cdot 10^{-2}$ M; glucose – $2.4 \cdot 10^{-3}$ M; KCl – $4.2 \cdot 10^{-2}$ M; $MgCl_2$ – $5 \cdot 10^{-3}$ M; NaF – $5 \cdot 10^{-2}$ M; ATP – $1 \cdot 10^{-3}$ M. Homogenate – 0.2 ml. Total volume – 0.5 ml)

Name of culture	Fall in glucose in $\mu g/10^6$ nuclei
Monkey kidney culture	10.5
HEP-2	3.2
MH	4.4

Recently Warburg has presented his ideas in relation to contemporary results and expressed them in the form of a completed theory [17]. According to Warburg, the specific characteristic of cancer metabolism is the destruction of respiration and a sharp increase in glycolytic activity. However, in the literature there is evidence that, for example, in embryonic tissue [18-20], in kidney marrow substance [21, 22], in leucocytes [23-26] and in some other normal tissues, metabolism in its qualitative respects resembles metabolism in cancer cells. Disagreeing with critics of this type, Warburg has shown in special experiments that "cancer" metabolism in normal tissues occurs only in the presence of injurious factors. When these tissues are incubated in serum which contains some quantity of lactic acid with assured presence of carbon dioxide in the gas medium, such an important indication of cancer metabolism as aerobic glycolysis, according to Warburg's data [27-29] is absent. However, tissue cultures certainly indicate that the metabolic characteristics described by Warburg are not specific properties of cancer cells only. It has long been known from the use of relatively primitive techniques of tissue culturing that normal cells grown in vitro are capable of high aerobic glycolysis [30-32].

In the preceding and present communications we have shown that normal cells cultured in vitro not only show high glycolysis but also have a number of disturbances in the respiratory apparatus. It is very important that the intense aerobic glycolysis observed when the tissue culture cells are incubated in the Warburg vessel is of the same order as the intensity of glycolysis in those vessels in which the cells were cultured, that is, under conditions which are clearly physiological for these cells. Hence, we cannot explain the presence of aerobic glycolysis by injury in this case. The presence in normal cells, cultured in vitro, of the "cancer" type of metabolism has, of necessity, been accepted by Warburg in very recent times [29]. Thus, we can confirm that high aerobic glycolysis and weakened respiration are not specific properties of tumor cells, but are inherent in a wider group of cells.

Similarities of metabolism in different tissue cultures. Until now we have not known of such biochemical differences as would allow us with sufficient accuracy to place one or another tissue culture in the class of normal or malignant tissue. The material which we have presented shows that the similarities in metabolism of cells from normal and cancer tissues are very deep. Besides, the requirements for a positive criterion of a alignancy in a tissue culture are very strict [33, 34]. Recently a number of such criteria have been proposed in the work of Leslie [12]. However, in our view, these criteria are not sufficiently well grounded. In particular, a high content of DNA in the cell nucleus would hardly be a sign of cancer, and it seems dangerous to us to class the MH cells on this basis as cancerous. Another criterion is apparently also little suitable, a higher aerobic glycolysis in normal cells than in cancer cells. It is not clear in which group we should place the MH cells which have an intermediate intensity of glycolysis. A third criterion, the ability of a culture of cancer cells as distinct from a normal culture to respond to the addition of insulin by an increase in glycolysis, cannot be considered specific until a greater number of cancer and normal tissues have been studied in this respect.

Significance of aerobic glycolysis. The tissue cultures permit us to draw some conclusions as to the significance of aerobic glycolysis. Recently Seits suggested a hypothesis according to which aerobic glycolysis is considered as the source of lactate which would seem to be an important energetic and plastic substrate for cancer cells [35]. However, such a theory evidently does not agree with the data obtained in tissue cultures. Our findings show that the addition of exogenous lactate results in its very weak utilization (if it is utilized at all) by the tissue culture cells. Beside this, the literature data [36] and also data from our laboratory indicate that ordinary tissue cultures do not have the ability to grow in media which contain lactate (or pyruvate) instead of carbohydrates.

It seems to us that the idea of those investigators who see the chief importance of aerobic glycolysis as supplying the cell with energy [17] and plastic substances [37, 38] are much more fundamental. Warburg made a calculation of the amount of energy obtained by the cell from respiration and from glycolysis. He found from

TABLE 7

Energetic Balance of Tissue Culture Cells

Name of culture	Amount of energy obtained from aerobic glycolysis, in %
Monkey kidney culture	24
HEP-2	25
MH	20

this that a molecule of formed lactate generates one molecule of ATP, while a molecule of used O_2 forms seven molecules of ATP. By this calculation the cancer cell obtains up to 50% of its energy from glycolysis [17]. We also made the corresponding calculation for tissue culture cells (Table 7). The calculation showed that, under the conditions of our experiments, cells of normal and cancer cultures obtained 20 to 25% of all their energy from glycolysis. These figures probably are low, since Warburg took the P:O ratio equal to be too great a value, 3.5.

Mechanism of the inverse Pasteur reaction. Any theory of the inverse Pasteur reaction should explain the resistance of this effect to the action of monoiodoacetate and should explain the removal of this effect in the presence of methylene blue [1, 13, 39], and should disclose why only a high concentration of glucose suppresses respiration, while a low glucose concentration does not change the oxygen requirement or even increases it [13, 40]. It seems to us that it is easiest to understand these various facts if we assume that the suppression of respiration is connected in some way with the functioning of the apotomous pentose phosphate path of assimilation of glucose and depends on the formation of reduced TPN in the dehydrogenation of glucose-6-phosphate and phospho-6-gluconate. The removal of the inverse Pasteur effect by methylene blue is explained by the acceptance of hydrogen from reduced TPN. From this point of view we can understand the occurrence of the inverse Pasteur effect in the presence of monoiodoacetate which evidently does not inhibit the initial steps of the apotomous path of glucose oxidation. It is interesting that in cells of an ascitic tumor with low glucose concentration, the assimilation of this substrate occurs almost exclusively by the Embden-Meyerhof scheme [13]. When the glucose concentration is raised, glycolysis is hardly increased [41] but the intensity of the apotomous oxidation of glucose rises markedly [13]. Strengthening the apotomous oxidation of glucose is thus accompanied by suppression of the demand for oxygen. This idea, in the end, must be confirmed experimentally.

SUMMARY

Normal cells cultured in vitro (cultures of monkey kidney cortex) and cancer cells (strain HEP-2), and also cells of a monkey heart strain were very similar in a number of important metabolic properties. In all these types of cells we found disturbance of the catalysts in the respiratory chain (both in the cytochrome and the succinic dehydrogenase portions). The inverse Pasteur effect was found in all three types.

There was very intense anaerobic and aerobic glycolysis in all the tissue cultures which we studied. In the normal cells of the monkey kidney culture the rate of assimilation of glucose under our conditions was the highest; in the HEP-2 cells it was the least, and in the monkey heart cells it occupied an intermediate position. The hexokinase activity changed in the same order.

The unusual combination of injury to the respiratory apparatus and high glycolytic activity is not a specific property of cancerous organisms alone, but is inherent in a wider group of tissues, especially normal and cancer cells grown in vitro.

LITERATURE CITED

[1] V.N. Gershanovich, V.I. Agol, R.N. Étingof and S.G. Dzagurov, Biokhimiya 23, 453 (1958).*

[2] H.W. Toolan, Cancer Research 14, 660 (1954).

[3] J.E. Salk, Amer. J. Publ. Health 47, 1 (1957).

[4] J.E. Salk and E.N. Ward, Science 126, 1338 (1957).

[5] D. Bodian, Virology 2, 575 (1956).

[6] J.E. Salk, J.S. Youngner and E.N. Ward, Amer. J. Hyg. 60, 214 (1954).

*Original Russian pagination. See C.B. translation.

[7] J.L. Melnick and E.M. Opton, Bull. World Health Organis. 14, 129 (1956).

[8] K.K. Sanford, W.K. Earle, et al., J. Nat. Cancer Inst. 11, 773 (1951).

[9] H.J. Phillips and J.E. Terryberry, Exptl. Cell. Res. 13, 341 (1957).

[10] I. Leslie, Nucleic Acids [Russian translation] (Izd. IL, Moscow, 1957) p. 7.

[11] A. Levan, Cancer 9, 648 (1956).

[12] I. Leslie, W.C. Fulton and R. Sinclair, Biochim. biophys. acta 24, 365 (1957).

[13] E. Racker, Ann. N.Y. Acad. Sci. 63, 1017 (1956).

[14] F. Seelich and K. Letnansky, Naturwissenschaften 44, 450 (1957).

[15] N.V. El'tsina and I.F. Seits, Doklady Akad. Nauk SSSR 77, 653 (1951).

[16] O. Warburg, Über den Stoffwechsel der Tumoren (Springer, Berlin, 1926).

[17] O. Warburg, Science 123, 309 (1956).

[18] D. Burk, Cold Spring Harbor Symp. 7, 420 (1939).

[19] R.J. O'Connor, Brit. J. Exptl. Pathol. 31, 390 (1950).

[20] C.A. Villee, Cold Spring Harbor Symp. 19, 186 (1954).

[21] P. György, W. Keller and T.Brehme, Biochem. Z. 200, 356 (1928).

[22] F. Dickens and H. Weil-Malherbe, Biochem. J. 30, 659 (1936).

[23] W.S. Beck and W.N. Valentine, Cancer Research 13, 309 (1953).

[24] W. Remmele, Acta haematol. 13, 103 (1955).

[25] I.S. Luganova, I.F. Seits and V.I. Teodorovich, Vopr. Med. Khimii 3, 428 (1957).

[26] F. Seelich, K. Letnansky, W. Frisch and O. Schnek, Z. Krebsforsch. 62, 1 (1957).

[27] O. Warburg, K. Gawehn and A.W. Geissler, Z. Naturforsch. 11b, 657 (1956).

[28] O. Warburg, K. Gawehn and A.W. Geissler, Z. Naturforsch. 12b, 115 (1957).

[29] O. Warburg, K. Gawehn and A.W. Geissler, Z. Naturforsch. 13b, 61 (1958).

[30] A.A. Krontovskii and I.A. Bronshtein, Zh. Éksp. Biol. i Med. 5, 1 (1926).

[31] A.A. Krontovskii and M.A. Magat, Vrach. Gazeta 19, 1393 (1930).

[32] O. Warburg and E. Kubowitz, Biochem. Z. 189, 242 (1927).

[33] Proc. Decen. Rev. Conf. on Tissue Culture, J. Nat. Cancer Inst. 19, No. 4 (1957).

[34] Cellular Biology, Nucleic Acids and Viruses (New York, 1957).

[35] I.F. Seits, Doklady Akad. Nauk SSSR 118, 344 (1958).•

[36] R.Sh. Chang and R.P. Geyer, Proc. Soc. Éxptl. Biol. Med. 96, 336 (1957).

[37] V.A. Éngel'gardt, Sessions of the Academy of Sciences USSR, on Peaceful Uses of Atomic Energy, July 1-5, 1955 [in Russian] (Izd. AN SSSR, Moscow, 1955) p. 80.

[38] N.V. El'tsina, Doklady Akad. Nauk SSSR 104, 436 (1955).

[39] R.N. Étingof and V.N. Gershanovich, Biokhimiya 18, 668 (1953).

[40] R.W. McKee, K. Lonberg-Holm and J. Jehl, Cancer Research 13, 537 (1953).

[41] L. Block-Frankenthahl and S. Weinhouse, Cancer Research 17, 1082 (1957).

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•See English translation.

THE MECHANISM OF THE ACTION OF SULFUR DIOXIDE AS A SUBSTANCE WHICH PROTECTS TRYPTOPHAN FROM DESTRUCTION IN ITS REACTION WITH SOME ALDEHYDES AND SUGARS

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We [1, 2] showed, simultaneously with foreign authors [3], that if hydrolysis of proteins is carried out by acids in an atmosphere of sulfur dioxide, less tryptophan is destroyed than when the atmosphere is air. It is interesting to study the mechanism of SO_2 action. For this purpose we carried out experiments with simpler systems, namely, solutions of pure tryptophan whose destruction was produced by some aldehydes, sugars, and other substances, and under these conditions we studied the protective action of sulfur dioxide.

In the first series of experiments we tried to give the quantitative characteristics of the tryptophan-destroying action of a number of aldehydes and sugars. The purpose of the second series of experiments was to explain the mechanism of the protective action of sulfur dioxide.

METHODS

We placed a definite amount of 0.5% tryptophan solution in 20% H_2SO_4 in a 20 ml ampoule and added Na_2SO_3 and some of the substance which could destroy tryptophan. As such substances we used: formaldehyde, acetaldehyde, benzaldehyde, furfural, acetone, glucose, fructose, galactose, sucrose, starch, inulin, and ascorbic acid. Na_2SO_3 was not added to the control samples. The ampoule was sealed, placed on a water bath, and this was boiled for four hours, after which the residual undestroyed tryptophan was determined by the Hopkins-Cole method, modified by Winkler [4]. Since SO_2 affects the determination of tryptophan, it was first removed by sucking off with a Komovskii pump and the passage of CO_2. This operation was repeated to full removal of SO_2, the absence of which was determined by reaction with zinc nitroprusside. We ran analogous experiments in which the ampoule was saturated with CO_2, N_2 and H_2S.

EXPERIMENTAL DATA AND DISCUSSION

In Table 1 we give data for those concentrations of substances which caused destruction of not more than 60% tryptophan.

We have consciously omitted data for higher concentrations (which cause deeper destruction of tryptophan), since then the remaining tryptophan could not be accurately determined for two reasons: first, the remaining tryptophan would give a very weak color, leading to great error, and second, colors from the decomposition products could be mixed with the color from tryptophan, which could distort the results considerably.

In order to determine quantitatively the tryptophan-destroying power of the substances which we studied, we submitted our data to mathematical treatment. We calculated the correlation coefficient [5] on the basis of the data in Table 1 and showed that for formaldehyde, ascorbic acid, fructose, starch, and inulin, the coefficient was 1.0; for furfural and benzaldehyde, 0.99, for sucrose, glucose and galactose, 0.97. Considering this, we used the method of least squares [6] to establish the interpolation equation for each substance separately (Table 2), from which we calculated the concentration of the substance which caused 50% destruction of tryptophan (this number was called C_{50}). We used this equation to calculate the lowest concentration of the substance which

TABLE 1

Degree of Destruction of Tryptophan Depending on the Added Substances and Their Concentrations. Composition of the mixture: 0.4 mg (0.2 mmoles/liter) tryptophan in 10 ml of 20% H_2SO_4; duration of boiling, 4 hours

Compound	Concentration, mmoles/liter	Destruction of tryptophan, %	Compound	Concentration, mmoles/liter	Destruction of tryptophan, %
Furfural	1.0	24.4	Fructose	1.3	15.5
	2.0	39.6		2.5	24.0
	3.0	56.2		5.0	48.2
Formaldehyde	0.5	27.0	Galactose	27.8	25.0
	0.75	40.0		55.5	41.8
	1.00	50.8		111.0	54.3
Benzaldehyde	2.0	18.5	Sucrose	3.0	32.0
	4.0	39.4		6.0	46.5
	6.0	48.0		12.0	56.2
Ascorbic acid	1.4	13.0	Starch	5.0	16.0
	2.8	24.0		10.0	21.5
	5.6	40.0		20.0	32.5
Glucose	56	28.6	Inulin	0.5	8.6
	112	37.0		1.0	28.7
	224	52.0		2.5	47.5
			Acetone	175	51.3

TABLE 2

Concentration of Aldehydes, Sugars and Other Substances Which Destroy 50% Tryptophan and the Minimum Concentrations Which Cause Complete Destruction of Tryptophan

Substance	C_{50}		C_{100}	
	mmoles/liter	g/liter	mmoles/liter	g/liter
Formaldehyde	1.0		2.0	
Acetaldehyde			5.5	
Furfural	2.6		5.7	
Benzaldehyde	8.6		24.2	
Ascorbic acid	7.3		15.7	
Fructose	5.3	0.95	11.2	2.12
Sucrose	9.0	3.08	28.7	9.81
Glucose	207.0	37.30	564.0	101.5
Galactose	92.0		251.0	
Acetone	175.0		400.0	
Starch		36.0		81.4
Inulin		2.56		5.36

would cause full decomposition of tryptophan (C_{100}). The results of the calculation are given in Table 2. As Table 2 shows, formaldehyde has the greatest tryptophan-destroying power; the substances studied for their tryptophan-destroying power could be divided into two groups. The first group, under the conditions of our experiment, caused destruction of 50% of the tryptophan at concentrations not above 10 mmoles/liter and its complete destruction at concentrations not above 30 mmoles/liter; it included formaldehyde, acetaldehyde, benzaldehyde, furfural, ascorbic acid, fructose, sucrose, and inulin. The second group consisted of glucose, galactose, acetone,

and starch. The destructive action of the second group was much lower: 50% destruction was produced only at concentrations not less than 90 mmoles/liter, that is, about ten times more than the concentration of the least active substance in the first group. It is interesting that fructose, sucrose and inulin on the one hand, and glucose, galactose and starch on the other, are sharply different in their destructive actions. According to the data in Table 2, the very great destructive action of fructose (surpassing even that of benzaldehyde) depends little on the presence of the keto group in it, since the destructive action of acetone is much less than that of fructose. Evidently, this action of fructose is related to its transformation under our experimental conditions into methoxyfurfural. We are justified in assuming that the tryptophan-destroying action of sugars is determined by the ease with which they can be changed to furfural or its derivatives. As experiments showed, those sugars which require more severe conditions to form furfural or its derivatives (in our experiments glucose, galactose and starch) have much less destroying action on tryptophan. Our assumption is also confirmed by the fact that, as Tilmans et al. showed, ascorbic acid in an acid medium is converted comparatively easily into furfural [7].

For a study of the mechanism of the protective action of SO_2 we carried out experiments analogous to those described above.

In the ampoule which contained a solution of tryptophan in 20% H_2SO_4 and the substance which destroyed tryptophan we added some quantity of sodium sulfite. The amount of H_2SO_4 added was increased to compensate for its partial neutralization. Sodium sulfite was not added to the control ampoules (we did not saturate the mixture directly with SO_2 since after boiling with H_2SO_4 this mixture acquired a reddish tint, which further hindered colorimetric determination of the remaining undecomposed tryptophan. For comparison we also studied the action of other gases, N_2, H_2S and CO_2. N_2 for saturating the ampoule was obtained by adding a concentrated solution of $NaNO_2$ to a hot solution of NH_4Cl. The gas was washed by passing it successively through a solution of H_2SO_4, alkaline permanganate and 5% pyrogallol. H_2S and CO_2 were obtained in the usual way and washed by passing through solutions of H_2SO_4 and pyrogallol.

The data given in Table 3 throw some light on the protective action of SO_2 in the hydrolysis of proteins in an atmosphere of this gas. Since in our experiments such inert gases as nitrogen and CO_2 protect tryptophan from destruction to about the same extent as does SO_2, we can consider that the protective action of SO_2 (in most cases), N_2 and CO_2 is not specific and is related to the removal of oxygen from the reaction sphere.

TABLE 3

Effect of SO_2, CO_2, N_2 and H_2S on the Preservation of Tryptophan When It is Boiled With 20% H_2SO_4 in the Presence of Some Sugars and Aldehydes. Duration of Boiling Four Hours. Amount of Sodium Sulfite 0.25 g

Decomposing substance	mmoles/liter	Amount of tryptophan, in mmoles/liter	Preservation of tryptophan in atm, %				
			air	SO_2	CO_2	N_2	H_2S
Glucose	50	0.4	65.0	84.0	98.7	–	–
"	50	0.6	65.0	87.5	91.2	–	–
Furfural	1	0.5	46.8	55.6	57.2	–	–
Ascorbic acid	1.4	0.6	87.5	100.0	99.0	55.5	–
Sucrose	5	0.5	43.6	55.8	–	50.4	–
"	5	0.5	40.8	51.2	–	–	–
"	5	0.5	47.0	63.0	70.3	–	–
"	5	0.5	42.8	54.3	–	–	37.2
Formaldehyde	1.75	0.5	0.0	73.0	0.0	–	–
"	1.0	0.5	0.0	81.2	0.0	–	–
"	0.67	0.5	61.7	91.8	63.7	–	–

It is difficult to believe that under the conditions of our reaction (strong acid medium) the protective effect of SO_2 can consist in its direct combination with the decomposing substance (binding the carbonyl group of the aldehyde?).

Gortner and Blish [8] have already shown that tryptophan is not destroyed when it is boiled in a solution of

a pure mineral acid, but is destroyed under the same conditions in the presence of carbohydrates, and have suggested that the destruction of the tryptophan is connected with condensation of the aldehyde groups with the imine group of tryptophan. Yoritaka [9] assumes that the destruction of tryptophan in the acid hydrolysis of casein is connected with the freeing of carbonyl groups which are then condensed with tryptophan.

However, SO_2 can also show a specific protective action toward certain substances (in our experiments, toward formaldehyde). This is shown by the fact (Table 3) that the protective action of SO_2 toward formaldehyde was very clear, while in this case under the same conditions CO_2 had no effect. In most cases CO_2 had a stronger protective action than SO_2. This is explained by the fact that under our conditions CO_2 produces a more complete removal of oxygen. We must remember that SO_2 can serve as an oxidant, being reduced to S under these conditions, as the work of Pedersen and Baker showed [3].

Our results confirm the role of oxygen as one of the important factors in the destruction of tryptophan. Monier and Jutisz [10] showed that the following factors were capable of destroying a considerable amount of tryptophan during hydrolysis of proteins by hydrochloric acid: presence of air; destruction caused by other amino acids; presence of impurities in the hydrochloric acid; temperatures at which the hydrolysis is carried out.

H_2S in our experiments showed a faster negative action, which was apparent, for example, in the greater destruction of tryptophan by sucrose in an atmosphere of H_2S than in ordinary (aerobic) conditions. Evidently, H_2S acts directly with tryptophan producing this decomposition. This problem demands special study.

Oxygen is not the only factor which activates the splitting of tryptophan by carbohydrates and aldehydes, especially when the splitting occurs in such a complex system as the protein hydrolysate. It is known that heavy metals can split amino acids. In the experiments of Dréze [11] ethylenediaminetetraacetic acid, which binds heavy metals, can preserve tryptophan in protein hydrolysis, though, it is true, in an alkaline medium. In an alkaline medium such reducing agents as $SnCl_2$ and cysteine keep tryptophan from destruction in the hydrolysis of proteins in the presence of starch.

The splitting of tryptophan during acid protein hydrolysis is due not only to its reaction with carbohydrates which enter the protein molecule, but also, as Olcott and Frenkel-Conrat showed [12], this process is connected with the reaction of tryptophan with other amino acids, especially with cystine or their derivatives (for example, pyruvic acid which is formed in protein hydrolysis from serine). The mechanism of the destruction of tryptophan under the influence of cystine consists in the oxidation due to the disulfide bridge of cystine, with the formation of an equivalent amount of cysteine. It has been shown that in this process there is more intensive destruction in the presence of oxygen than under anaerobic conditions.

We may remark that tryptophan is not split in all cases under the influence of aldehydes with formation of humins (darkening of the solution and formation of a brown-black precipitate). Thus, intense destruction of tryptophan under the influence of benzaldehyde does not cause darkening of the solution. An analogous effect has been found by Olcott and Frenkel-Conrat [12] who did not observe formation of humins when tryptophan was split under the influence of cystine.

SUMMARY

We have studied the decomposing action on tryptophan of some aldehydes (furfural, formaldehyde, acetaldehyde, benzaldehyde), sugars (glucose, fructose, galactose, sucrose, starch, inulin), acetone, and ascorbic acid reacting in a 20% sulfuric acid medium during four hours at 100°, and also the mechanism of the action of SO_2, a substance which under these conditions keeps tryptophan from destruction.

We have established that, together with the aldehydes, only those sugars have a decomposing action which are ketohexoses. The decomposing action of aldohexoses (we studied glucose and galactose) is about 20-40 times weaker than that of fructose. The conclusion follows that the tryptophan-destroying action of sugars is chiefly determined by the ease with which they are converted to furfural or its derivatives. The decomposing action of ascorbic acid is of the same order as that of fructose, and that of acetone, of the order of glucose.

We have shown that SO_2, to a definite degree, prevents tryptophan from decomposing by the above-mentioned substances, but the protective action of SO_2 is basically nonspecific, since the same action is produced by the inert gases CO_2 and nitrogen, if the reaction of tryptophan with these substances occurs in them. The action of SO_2, N_2 and CO_2 is connected with the establishment of anaerobic conditions in the medium, which to some degree hinders the decomposition of tryptophan.

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LITERATURE CITED

[1] Yu.N. Kremer, Doklady Akad. Nauk SSSR 98, 627 (1954).

[2] A.A. Shmidt and Yu.N. Kremer, Sb. Zdravookhr. Sov. Latvii No. 11, 16 (1954).

[3] J.W. Pedersen and B.E. Baker, J. Sci. Food and Agric. 5, 549 (1954).

[4] R. Block and D. Bolling, The Amino Acid Composition of Proteins and Food Products [Russian translation] (Izd. IL, Moscow, 1949) p. 116.

[5] E. Weber, Grundrisz der biol. Statistik (VEB Gustav Fischer Verlag, Yena, 1957) p. 292.

[6] K.P. Yakovlev, Mathematical Treatment of the Results of Measurements [in Russian] (Gosizdat, Techn.-Teoretiches. Lit., Moscow, 1957) p. 358.

[7] I. Tillmans et al., Z. Untersuch. Lebensm. 63, 1 (1932) cited by F. Smith, The Vitamins (Academic Press, New York, 1954) p. 181.

[8] R.A. Gortner and M.I. Blish, J. Amer. Chem. Soc. 37, 1915 (1930).

[9] T.Z. Yoritaka, Z. physiol. Chem. 270, 41 (1941).

[10] R. Monier and M. Jutisz, Bull. Soc. chim. biol. 32, 208 (1950).

[11] A. Drèze, Arch. internat. physiol. 60, 200 (1952).

[12] H.S. Olcott and H. Frenkel-Conrat, J. Biol. Chem. 171, 583 (1947).

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THE SYNTHESIS OF PHENYLALANINE AND TRANSFORMATIONS OF PHENYLPYRUVIC ACID IN THE RIPENING WHEAT EAR

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The ripening wheat ear is an organ with definite synthetic powers. Therefore, it is an excellent object for in vivo studies of the pathways for the synthesis of different compounds whose possible precursors are drawn into the ear from the transpiration current through the stem [1, 2].

Kizel' [3] showed that in the ripening rye ear there are large amounts of phenylalanine, which he separated preparatively and identified by various derivatives. Therefore, we can assume that in the ripening ear there was energetic synthesis of phenylalanine.

The problem of the present study is to investigate the synthesis of phenylalanine in the ripening wheat ear from phenylpyruvic acid, which is the nearest nitrogen-free precursor of phenylalanine.

MATERIALS AND METHODS

The work was carried out with a beardless wheat hybrid No. 186 from the field station of the Timiryazev Agricultural Academy. The first two experiments were run with wheat in the early middle phase of milky maturity, and the third experiment at the end of the milky phase and the beginning of the waxy stage. The heads were quite different in appearance: the first were greener, the second, more yellow. The material collected in the field was brought to the institute in moist paper and made up into portions of the desired size, with an attempt to prepare average samples. In each sample twelve heads were used. The stems of the heads were cut under water so that there would be no air bubble in the conducting pathways and were then placed in 0.05 M solutions of ammonium and sodium salts of phenylpyruvic acid. These solutions were prepared by neutralizing the free phenylpyruvic acid with 8% solutions of NH_4OH or NaOH. The controls were samples with water. A fourth sample of the ears was placed in 0.05 M NH_4Cl solution. In each sample we used 30 ml of the corresponding solution. The solutions in which the stems of the ears were immersed were placed in an ice bath to avoid development of microbes in them. Absorption of the experimental solutions took a day, after which the ears were quickly broken up into separate pieces and carefully mixed. The central stalk was removed but the scales were kept. A 20 g sample was taken from each part of the material and treated with 70 ml of freshly distilled 96% ethyl alcohol. The samples were kept in this state in vessels with ground-glass stoppers in a cold room at 3° for one to two months, and then submitted to further treatment. The alcohol was pipetted as completely as possible from the samples and transferred to another vessel. The amount was measured; it varied in different experiments from 53 to 55 ml. The amount of alcohol withdrawn by the pipet from each sample of one experiment was the same. The ears were placed in a Petri dish and dried at room temperature for a day. Then each sample was divided into four parts by weight and each portion was ground in a mortar for eight minutes. The ground material was transferred quantitatively by 96% alcohol into a vessel with a ground-glass stopper and the original portion of alcohol drawn from the material was added. The total volume of alcohol in each sample was 140 ml, so that the final concentration of alcohol in the samples was about 90%. The vessel with the fixed material was wrapped in cellophane and kept in a cold room.

The fixed samples were studied for their content of phenylalanine, alanine, glutamic acid, tyrosine, γ-aminobutyric acid, glutamine, valine, threonine, serine, and leucine and isoleucine. In the determination of the amino acids we used the method of quantitative paper chromatography in the modification which we have described before [4].

For the analysis we took a definite amount (14 or 28 ml) of filtered alcoholic extract and evaporated it dry in a vacuum at 40-45°. To the dry residue we added 1 ml of H_2O, then all the samples were centrifuged at the same time for 15 minutes at 2000 rpm, and filtered through a small filter. Without the preliminary centrifuging the filtration went very slowly. The clear solution was placed on paper. First we established how much solution had to be added to the paper for best determination of the amino acids. Here two conditions had to be fulfilled: the amount should not be too great (then the amino acids would be poorly separated from each other and would not conform to the Lambert-Beer law) but at the same time it should not be too small (in this case, when the spots of amino acid were eluted the color of the solutions would be very weak and the difference between the experimental and control, visible to the eye, would not register on the photoelectric colorimeter). If we used 14 ml of alcoholic extract, then the amount of evaporated extract deposited on the chromatogram in the determination of the different amino acids was from 0.02 to 0.12 ml. An especially large amount of extract had to be deposited in the determination of tyrosine and γ-aminobutyric acid, since when they were determined in a mixture of m-cresol-phenol-borate buffer, pH 8.3 (4:2:1) they gave a very weak color with ninhydrin. In the mixture o-cresol-phosphate buffer, pH 6.2, where γ-aminobutyric acid reacts more intensely with ninhydrin, these two amino acids are not separated. The mixture m-cresol-phenol-borate buffer, pH 8.3 was also used in the determination of glutamic acid, serine and threonine.

The determination of phenylalanine, alanine, glutamine, valine, and leucine with isoleucine was carried out in a mixture of o-cresol-phosphate buffer, pH 6.2 (1:1) (the lower layer was used), and for the separation of glutamine and alanine this mixture was passed a second time, after drying the chromatogram for 20 minutes at 60°. As the o-cresol chromatogram showed, the results of this double passage of the solvent gave a spot above the phenylalanine which contained not only leucine but also isoleucine (upper part of the "leucine" spot). Also, in the first five minutes of developing the chromatogram it became evident that, at the same place as that of the leucine, there was a spot of an unknown compound which was colored bluish gray by ninhydrin. When the chromatogram was further developed, the violet spot of isoleucine covered the spot of this unknown compound. Since, even with double passage of the o-cresol, complete separation of leucine, isoleucine and the unknown compound was not obtained, we determined their mixture and called it the amino acids of the leucine group. As a standard solution for the determination of these amino acids we used a solution of leucine. In the rest of this paper we will use the following abbreviations in describing the experimental results: sodium phenylpyruvate Na-PP, ammonium phenylpyruvate NH_4-PP, phenylalanine PA.

EXPERIMENTAL RESULTS

The results of the determination of phenylalanine are given in Table 1.

From the data presented in Table 1 it is evident that in ripening ears there is an especially intense synthesis of PA from phenylpyruvate. The synthesis occurs especially energetically in the less mature ears; in them the increase in amount of PA reaches 630% with respect to the control experiments with water. In the more mature ears the increase in PA reaches 300% compared to the water control. The synthesis of PA in less mature ears in wheat is seen on the o-cresol chromatograms shown in Fig. 1.

TABLE 1

Phenylalanine Content in the Ears

Expt. No.	Maturity of ear	Content in μg/1 g dry weight of ear which imbibed				Increase in PA compared to controls where H_2O was imbibed, %		Increase in PA based on % imbibed NH_4-PP compared to imbibed Na-PP
		NH_4-PP	Na-PP	NH_4Cl	H_2O	NH_4-PP	Na-PP	
1	Less mature	801.7	660.4	127.5	109.8	630.1	501.5	21.4
2	The same	717.0	613.3	101.8	95.1	653.9	544.9	16.9
3	More mature	276.3	218.4	69.3	67.4	309.9	224.0	26.5

A very important conclusion, which is clearly evident from our data, is that the addition of NH_4-PP greatly stimulates the synthesis of phenylalanine (by 17-26%) compared to experiments in which Na-PP was added to the ears. This increase is visible in the chromatograms of Fig. 1.

This suggests that PA synthesis in the ears can occur not only by the enzymatic reaction of transamination, which, as we showed earlier [4], occurs in pea-sprout homogenates, but also by direct amination of phenylpyruvic acid by ammonia. This question requires further, more detailed study.

In all the experiments on chromatographing the extracts in mixtures of o-cresol-phosphate buffer, pH 6.2, it was established that the addition of phenylpyruvic acid to the ears, in addition to the synthesis of PA, caused a sharp increase in the intensity of spots formed from γ-aminobutyric acid and tyrosine. This is clear in the chromatograms of Fig. 1.

As we have already shown, for the quantitative separation of tyrosine and γ-aminobutyric acid we must use the chromatographic mixture m-cresol-phenol-borate buffer, pH 8.3. The results of the determination of tyrosine are given in Table 2.

TABLE 2

Content of Tyrosine in the Ears

Expt. No.	Maturity of the ear	Content in μg/1 g dry weight of ear, in which was imbibed				Increase in tyrosine in % compared to control where H_2O was imbibed	
		NH_4-PP	Na-PP	NH_4Cl	H_2O	NH_4-PP	Na-PP
1	Less mature	151.5	121.5	67.0	64.9	133.4	87.2
2	" "	148.3	152.7	107.6	89.2	66.3	71.2

From the data given in Table 2 it is clear that in the less mature ears there is synthesis of tyrosine from phenylpyruvic acid. However, this synthesis does not go as intensively as the synthesis of PA, to judge by the qualitative o-cresol chromatograms, where the synthesis of tyrosine is also observed. Although in these chromatograms tyrosine and γ-aminobutyric acid occur together, at first only tyrosine reacts with the ninhydrin developer, giving a gray-brown color. After two to three minutes from the beginning of the development of the chromatogram it is evident that the tyrosine spot in the sample which imbibed NH_4-PP and Na-PP is 1.5-2 times as strong as in the samples which absorbed NH_4Cl and H_2O.

Quantitative determination of tyrosine in the more mature ears does not occur. Thus, the ripening wheat ear has the ability to transform phenylpyruvic acid into tyrosine.

A very interesting question is that of the mechanism of enzymatic hydroxylation of the benzene ring leading to the formation of tyrosine. Does hydroxylation occur at the stage of phenylpyruvate or is the hydroxyl group introduced into PA?

The answer to this question can be obtained only by further investigation.

As to γ-aminobutyric acid, the results of its quantitative determination are given in Table 3.

From the data of Table 3 it is perfectly clear that γ-aminobutyric acid is formed in the ears from phenylpyruvic acid which is added.

Thus, our results not only agree with the data of Carles [5] that free γ-aminobutyric acid plays an important part in amino acid and protein metabolism of the wheat plant, but also, it seems, indicate that in the tissues of the ear there is a deep decomposition of the phenylpyruvic acid with formation of γ-aminobutyric acid.

In the earlier phases of ripening of the ear the predominance of phenylpyruvic acid is accompanied by accumulation in the ear of γ-aminobutyric acid and also a number of other amino acids, namely the acids of the leucine group, valine, threonine, and to a smaller degree, alanine. The synthesis of the amino acids of the leucine group and also valine is clearly shown on the chromatograms of Fig. 1. From these chromatograms we see

TABLE 3

Content of γ-Aminobutyric Acid in the Ears

Expt. No.	Maturity of the ear	Content in μg/1 g dry weight of ear, which imbibed				Increase in γ-aminobutyric acid in % of control where H_2O was imbibed	
		NH_4-PP	Na-PP	NH_4Cl	H_2O	NH_4-PP	Na-PP
1	Less mature	306.0	175.3	111.5	99.0	209.1	77.1
2	" "	147.2	176.6	91.9	73.1	101.4	141.6
3	More mature	99.0	84.6	55.4	58.3	69.8	45.1

that in the leucine group there is an increase in the upper and lower parts of the spots. Thus, also leucine and isoleucine are synthesized with about the same intensity. This finding confirms the data from the almost quantitative determination of them in the o-cresol chromatograms obtained by double passage of the solvent.

As to the unknown compound found in the leucine group, it is clear from the initial development of the chromatogram that its synthesis also occurs from NH_4-PP and Na-PP.

TABLE 4

Content in the Ears of Alanine, Serine, Valine, Threonine, and Amino Acids of the Leucine Groups (Leucine, Isoleucine + Unknown Compound)

Expt. No.	Maturity of the ears	Amino acid	Content of amino acid in μg/1 g dry weight of ear in which was imbibed				Change in content of amino acid compared to imbibation of H_2O	
			NH_4-PP	Na-PP	NH_4Cl	H_2O	NH_4-PP	Na-PP
1	Less mature	Leucine group	422.7	374.5	187.3	278.2	+51.9	+34.6
		Threonine	62.3	62.3	52.4	49.2	+26.6	+26.6
		Valine	240.8	243.5	152.5	198.0	+21.6	+23.0
		Alanine	593.9	540.4	460.1	492.2	+20.7	+ 9.8
2	The same	Leucine group	359.8	363.9	206.0	232.8	+54.6	+56.3
		Valine	229.9	225.9	169.0	173.4	+32.6	+30.3
		Threonine	45.5	48.2	40.1	37.5	+21.4	+28.5
		Serine	56.2	56.2	56.2	56.2	0	0
3	More mature	Serine	34.3	30.9	48.3	62.9	−45.5	−50.9
		Threonine	28.3	24.8	35.0	36.8	−23.1	−32.6
		Alanine	357.5	306.6	399.9	405.0	−11.7	−24.3
		Valine	108.4	106.7	123.7	126.2	−14.1	−15.5
		Leucine group	173.3	155.5	148.3	180.1	− 3.8	−13.7

However, in the later phases of ripening of the ear tissues there is a definite loss of ability for such deep transformations of the phenylpyruvate molecule, and here drop out the first reactions of enzymatic transamination of phenylpyruvate with loss of serine, threonine, alanine, and to a lesser degree, valine and the amino acids of the leucine group. This is clearly shown in the results of Table 4 where we also give the figures for the increase in a number of amino acids when phenylpyruvate is imbibed by less mature wheat ears. It also follows from these results that in less mature ears there is no increase in content of serine due to phenylpyruvate. It is important to observe that on the o-cresol chromatogram the alanine spot evidently also contains some compound which reacts with ninhydrin somewhat more slowly than alanine.

As is known, a number of studies carried out by the isotope method [6] showed that PA, tyrosine, and p-hydroxyphenylpyruvic acid in the animal organism are split with rupture of the benzene ring and formation of four carbon compounds, fumaric and acetoacetic acids. If such a process also occurs in plants, then it is easy to assume that further transformations of these compounds can lead to the synthesis of the amino acids which we found in our experiments in the earlier phases of the ripening of the ears. An important problem for further study is to explain the exact enzyme mechanism which in plant organisms controls the splitting of the benzene ring of phenylpyruvic acid and PA.

All these results are also interesting from a more general viewpoint. They indicate that as the ears ripen, there take place in them not only quantitative, but also deep qualitative changes in metabolism especially in the change in the nature of enzymatic transformations of amino acids.

Since in plant tissues glutamic acid takes a particularly active part in enzymatic transaminations [7], it is natural that we should follow its changes in the material which we have studied. The results are given in Table 5.

TABLE 5

Content of Glutamic Acid in the Ears

Expt. No.	Maturity of ear	Content in μg/1 g dry weight of ear in which was imbibed:				Loss of glutamic acid in % compared to controls where H_2O was imbibed		Increase in glutamic acid in % when NH_4-PP is imbibed compared to imbibation of Na-PP
		NH_4-PP	Na-PP	NH_4Cl	H_2O	NH_4-PP	Na-PP	
1	Less mature	49.2	32.4	83.1	68.1	27.8	52.9	51.9
2	" "	54.9	46.8	88.3	92.3	40.5	49.3	17.3
3	More mature	17.6	15.2	44.1	48.1	63.4	68.4	15.6

According to the data of Table 5 in all the experiments on the introduction of phenylpyruvic acid into the ears there is a considerable loss in glutamic acid content, which is used in transamination with phenylpyruvate. It is also evident that when the ear imbibes NH_4-PP the content in the ear of glutamic acid is not as strongly decreased as when Na-PP is imbibed. This is evidently because when NH_4-PP is introduced into the ear there is also use of glutamic acid in its transamination with phenylpyruvate and the synthesis of glutamic acid by the reaction of direct reductive amination of ammonium α-ketoglutarate [8].

TABLE 6

Content of Glutamine in the Ears

Expt. No.	Maturity of the ear	Content in μg/1 g of dry weight of ears which imbibed				Loss of glutamine in % of control where H_2O was imbibed		Increase in glutamine in % due to NH_4Cl compared to H_2O
		NH_4-PP	Na-PP	NH_4Cl	H_2O	NH_4-PP	Na-PP	
1	Less mature	133.8	96.6	342.6	171.3	21.9	43.6	100.0
2	" "	100.4	107.6	378.5	179.5	44.1	40.1	110.9
3	More mature	81.5	71.5	242.1	130.0	37.3	45.0	86.2

The determined content of glutamine in the ears given in Table 6 shows that when phenylpyruvate is introduced into the ears there is a considerable fall in glutamine, which evidently takes part in the transamination reaction [9]. The absolute amount of glutamine used even surpasses the amount of glutamic acid used. Thus, it is clear that in the living plant tissue glutamine can have an even more active amino group in the reaction of transamination with ketoacids than free glutamic acid. This fact once more indicates the great mobility and extremely important role of glutamine in nitrogen metabolism of plants. It is also very apparent from the data on the quantitative content of glutamine that when there is a lack of carbohydrate nutrients and an excess of ammonia,

when a solution of NH_4Cl is added to the ears, glutamine is the compound which separates and binds the excess ammonia which enters the tissues. These results agree with those of experiments carried out on other plants using the isotope N^{15} [10] which showed the very high metabolic activity of glutamine as a compound which binds the ammonia entering the plants. The change in glutamine content is clearly visible in the chromatograms shown in Fig. 1.

SUMMARY

Using quantitative paper chromatography we have studied the synthesis of phenylalanine and the transformations of other amino acids in ripening wheat ears into which the ammonium or sodium salts of phenylpyruvic acid were introduced through the stem by the transpiration current.

The added phenylpyruvate caused a synthesis of phenylalanine which reached 630% of the control experiments with water. When ammonium phenylpyruvate was added to the ears, the synthesis of phenylalanine was 17-26% more energetic than when sodium phenylpyruvate was used. In addition to phenylalanine, the wheat ears synthesized tyrosine from phenylpyruvate, but less intensely, and there was energetic formation of γ-aminobutyric acid.

In the earlier phases of the ripening of the ears when phenylpyruvate was introduced there was synthesis of the leucine group of amino acids and also of valine, threonine, and alanine. In the later phases of ripening the ears lost the ability to transform phenylpyruvate into these amino acids which apparently were themselves used in the process of transamination with phenylpyruvate.

The synthesis of phenylalanine when phenylpyruvate was introduced into the ears was accompanied by expenditure of glutamic acid and glutamine which reacted by transamination with phenylpyruvate. When ammonium chloride was introduced into the ears, there was intensive synthesis of glutamine which in this case played the part of a compound which could bind the ammonia entering the ears.

LITERATURE CITED

[1] V.L. Kretovich and Z.G. Evstigneeva, Doklady Akad. Nauk SSSR 66, 429 (1949).

[2] V.L. Kretovich and V.I. Yakovleva, Doklady Akad. Nauk SSSR 116, 455 (1957).*

[3] A.R. Kizel', S.G. Navashina Collection [in Russian] (Izd. Gos. Timiryazevskogo N.-I. Inst., Moscow, 1928) p. 49.

[4] V.L. Kretovich and Zh.V. Uspenskaya, Biokhimiya 23, 248 (1958).**

[5] J. Carles, Compt. Rend. Acad. Sci. 244, 110 (1957).

[6] A. Meister, Biochemistry of the Amino Acids (Academic Press, New York, 1957).

[7] V.L. Kretovich, Izv. Akad. Nauk SSSR, Ser. Biol. No. 2, 129 (1958).

[8] V.L. Kretovich, A.A. Bundel' and V.I. Gunar, Ukr. Biokhim. Zh. 27, 342 (1955).

[9] L.S. Olenicheva, Biokhimiya 20, 165 (1955).

[10] V.L. Kretovich, Z.G. Evstigneeva and E.G. Plyshevskaya, Doklady Akad. Nauk SSSR 109, 1001 (1956).

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*Original Russian pagination. See C.B. translation.

**See English translation.

SENSITIVE AND RAPID METHOD FOR DETERMINATION OF CITRIC ACID IN BIOLOGICAL MATERIAL

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It has been shown by us in an earlier publication that electrolysis of citric acid in sulfuric acid solution containing bromide ions, and using lead anodes, leads to the formation of pentabromoacetone (PBA). On this we based a method for the determination of microquantities of citric acid in biological materials and drugs [1]. In the present paper we describe a modification of this method, adapted to the determination of citric acid in blood and urine.

EXPERIMENTAL

A special feature of our proposed method is that it makes use of an electrolytically generated oxidant.

We found that not even traces of PBA were formed during electrolysis of citric acid in a bromide-containing electrolyte when smooth platinum electrodes were used. Evidently the oxygen overpotential developed in such systems is insufficient to ensure oxidation of citric acid to readily brominated acetonedicarboxylic acid.

Addition to the anolyte of Mn^{2+} ions leads to their conversion into Mn^{3+} and Mn^{4+} ions [2, 3]. In view of their high oxidation potential, these ions react with citric acid to give acetonedicarboxylic acid, from which PBA is then formed. With amounts of citric acid not exceeding 40 μg, the entire sequence of reactions is completed within ten minutes, thanks to the presence of the oxidant ions which bring about the initial phase of the reaction in the bulk of the anolyte.

After completion of electrolysis the manganese ions present in the reaction mixture are reduced to the manganous form, and free bromine is eliminated.

PBA is then extracted from the colorless reaction mixture, and a color reaction is developed by heating the extract with pyridine and alkali. Under such conditions a pink to red coloration develops, the intensity of which is proportional to the PBA concentration [4].* A blank not containing citric acid remains colorless when similarly treated.

The optical density of the colored solutions was measured in a SF-4 spectrophotometer, the solutions being first clarified by addition of an acetone-ammonia mixture. Measurements may also be performed using an FM photometer, but the sensitivity and precision attainable are smaller.

The optical density of the colored solutions obtained is proportional to the citric-acid concentration, over the range 10-40 μg.

The yields of PBA given by the electrochemical process amount to 96-98% of the theoretical amount expected from citric acid.

Construction of a Calibration Curve

Into the electrolytic cell of the special equipment (Fig. 1) are placed not more than 4 ml of test solution, containing 10 to 40 μg of citric acid, 0.5 ml of 20 N H_2SO_4, 0.1 ml of 8% $MnSO_4$, 0.2 ml M of KBr, and 0.25 ml of 40% H_3PO_3 [3].

*The reaction of pyridine with pentabromoacetone was discovered by us in 1950-1951, but we did not publish our finding. This reaction was used by Ettinger in 1952, but he did not enter into its chemistry.

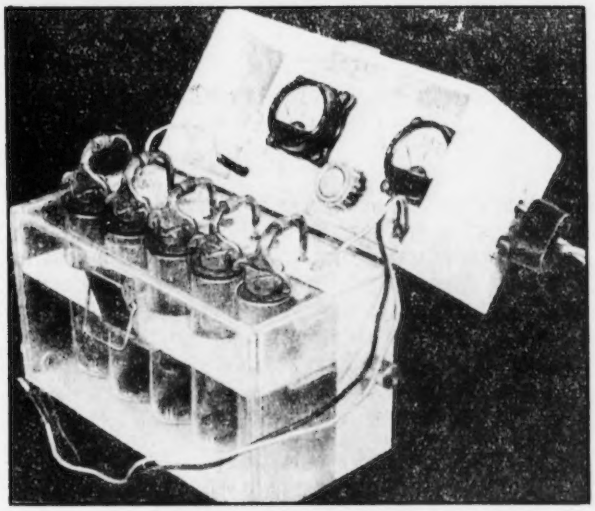

Fig. 1. Photograph of the equipment during the course of an analysis. The anode cells containing the electrodes are to be seen above the casing. The upper part of the equipment – the "lid" – contains an ammeter (on the left), and a voltmeter (on the right). Between them is a knob controlling the rheostat. The plug connecting the instrument with a dc source is shown on the right of the cover (the leads are marked + and –).

The volume of anolyte was made up to 5 ml, the cell was placed in position, and a platinum electrode was inserted (the part of the anode immersed in the solution measured 2.5 x 1.1 cm, thickness 0.02 cm); the current was then switched on for ten minutes (dc 0.2 A at 3.0 v, average current density about 4 A/dm^2). After completion of electrolysis the cell was removed from the instrument, together with the anode, and the anolyte was treated with 0.5 ml of 1% hydrogen peroxide and 0.2 ml of 12% sulfosalicylic acid, in order to eliminate free bromine and higher oxides of manganese. The completely colorless anolyte was transferred quantitatively to a glass-stoppered tube, capacity 20 ml. The anode vessel and the anode were washed with 6 ml of iso-octane ("Pure" or "Analytical" grades), and the washings were transferred quantitatively to the tube containing the anolyte, which was then shaken energetically for one minute.

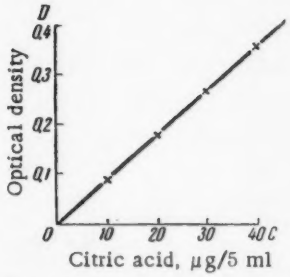

Fig. 2. Relationship between concentration of citric acid in the anolyte and the optical density of the colored solutions obtained.

Five ml of the supernatant was transferred to a second tube containing 2 ml of 30% KOH and 4 ml of pyridine.

For the development of the color reaction, the contents of the tube were shaken energetically, and the tube was placed for 3-4 minutes in a water bath at 80°, and then in cold water.

The presence of citric acid in the sample was shown by the appearance of a faint pink to red color. Control systems to which citric acid had not been added should be quite colorless after similar treatment with 3 ml of pyridine and 2 ml of acetone-ammonia mixture (4:1). Optical densities were read at 530 mμ.

The coloration fades gradually, for which reason the readings should be made within 5-10 minutes of color development.

When serial determinations are being done it is best to begin with the more faintly colored solutions. The stability of the coloration is somewhat enhanced by addition of a drop of 30% KOH before taking the readings.

The necessary standards were prepared from the control solutions.

Each point shown on the calibration curve (Fig. 2) is the mean of three determinations.

Determination of Citric Acid in Biological Material

Special experiments showed that the following substances, added in amounts of 100 μg to the anolyte and subjected to electrolysis, did not give any color reaction with pyridine: arginine, ascorbic acid, glycerophosphoric acid, glutamic acid, levulinic acid, maleic acid, lactic acid, butyric acid, uric acid, nicotinic acid, pyruvic acid, thiocyanic acid, quinic acid, malonic acid, acetic acid, malic acid, phenylpyruvic acid, adrenaline, acetaldehyde, alloxan, histidine, glycine, glucosamine, guanidine, inositol, urea, caffeine, xanthine, creatine, creatinine, thiamin, pyrocatechol, glucose, phenylalanine, tryptophan, ethanol, glycerol and phenol. A coloration developed in the presence of high concentrations of acetoacetic acid and of β-hydroxybutyric acid.

Determination of Citric Acid in Biological Materials

Material	Citric acid			
	found in sample,	added to sample,	total content	
	μg	μg	in μg	in %
Blood (dog)	6.3	–	6.3	
"	6.3	5.5	11.7	99.4
	13.5	–	13.5	
	13.5	2.25	15.6	98.9
Urine (human)	10.5	–	10.5	
"	10.5	10.0	20.7	101.2
	15.8	–	15.8	
	15.8	5.0	20.6	99.1
Urine (rat)	11.3	–	11.3	
	11.3	10.5	21.3	97.8
	7.2	–	7.2	
	7.2	15.0	22.3	100.5

Should the concentration of these acids be raised, as occurs in some pathological conditions, they are eliminated by cautious boiling of the urine, before proceeding to determination of citric acid.

The specificity of the method is thus such, that it may be applied in the presence of all of the above-enumerated substances.

The samples are deproteinized by a standard tungstic acid procedure. Trichloroacetic acid cannot be used for precipitation of protein, as it gives colored products with pyridine and alkali.

We found that about 30 minutes was needed for the analysis of five samples. The method is thus one of the most rapid ones known, while being highly sensitive, and sufficiently specific. The precision and the reproducibility of the results are up to the standard of photometric methods (see Table).

Examples of analyses: 1. Blood (dog). Heparinized blood (0.5-1.0 ml) was deproteinized with sodium tungstate [5]. The protein-free filtrate is cautiously evaporated down to 4 ml. The whole of the residue, or an aliquot thereof, depending on the expected citric-acid content, is transferred to the anode cell.

2. Urine (human). A 1:10 dilution of the urine is prepared, and one ml is placed in a centrifuge tube, followed by about three drops of 20 N H_2SO_4, 0.25 ml of 10% sodium tungstate, and water to 5 ml, and the mixture is shaken and centrifuged. From 0.25 to 0.5 ml of supernatant are taken for analysis.

3. Urine (rat). The urine is diluted 1:10 as before, 1 ml is placed in a centrifuge tube, deproteinizing reagents are added as before, and water to 4 ml, and the whole of the centrifugate is taken for analysis.

The analytical results are presented in the Table.

SUMMARY

A rapid method serving for the determination of amounts of from 10 to 40 μg of citric acid is described. The method is based on conversion of citric acid into acetone by an oxidant generated in the anolyte, with simultaneous bromination to pentabromoacetone, the amount of which is estimated from the coloration given with pyridine and alkali.

The specificity of the method permits of its application to the determination of citric acid in blood and urine.

LITERATURE CITED

[1] A.P. Safronov, Zhur. Analit. Khim. 13, 360 (1958).

[2] I. Guben, Methods of Organic Chemistry, Vol. 2 [in Russian] (GONTI, Chem. Lit., Moscow-Leningrad, 1941).

[3] C.A. Jacobson, Encyclopedia of Chemical Reactions, v. IV (Reinhold, New York, 1951).

[4] R.H. Ettinger, L.R. Goldbaum and L.H. Smith, J. Biol. Chem. 199, 531 (1952).

[5] V.S. Asatiani, Methods of Biochemical Research [in Russian] (Medgiz, Moscow, 1956).

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NEW METHOD FOR STAINING PROTEIN ELECTROPHORETOGRAMS

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The study of the distribution of plasma proteins has been found to be of considerable prognostic value in a number of diseases, in permitting an appraisal of the course of the morbid process and development of the defense mechanisms of the organism. The electrophoretic method for separation of plasma proteins on a paper strip has, very justifiably, received wide application in clinical pathology laboratories, in view of the relative simplicity of the equipment and procedures, and also of the small amounts of material required for the assay.

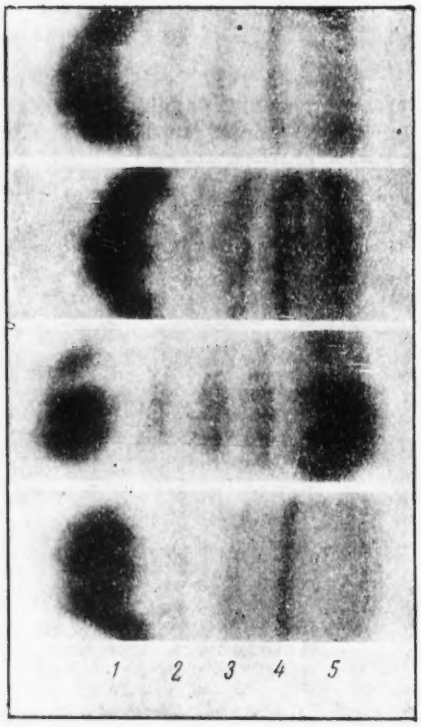

Fig. 1. Electrophoretograms of serum proteins, stained with acid chrome dark blue: 1) albumin; 2) α_1-globulin; 3) α_2-globulin; 4) β-globulin; 5) γ-globulin.

The most widely used stain, both in the Soviet Union and abroad, is bromphenol blue in saturated mercuric chloride solution [1-4]. The use and storage of strong solutions of mercuric chloride is not always convenient in clinical laboratories.

We have applied a new dye for staining protein electrophoretograms – acid chrome dark blue, which is manufactured by Soviet factories for use as an indicator for determining the hardness of water. This dye gives rapid and distinct staining of protein fractions on paper electrophoretograms. Excess of dye is readily removable from the paper. The stained electrophoretogram does not fade after long storage. Acid chrome dark blue can be rapidly and quantitatively extracted from the paper by alkaline solutions.

Electrophoretic separation of proteins was effected in a mixed veronal-borate buffer of pH 8.6, ionic strength 0.06, with a potential gradient of 8-10 V/cm, current strength 2.0 to 2.5 mA per strip, using highly absorbent chromatographic paper, produced by the 2nd Leningrad Paper Factory. Separation of serum proteins into five fractions (albumin, α_1-, α_2-, β-, and γ-globulins) was achieved after five hours of electrophoresis at room temperature (Fig. 1).

Staining of electrophoretograms. We used a 0.08% solution of acid chrome dark blue in 10% acetic acid. The strips were dipped in the dye solution in photographic dishes for a period of 12-15 minutes. A volume of 250 ml of dye solution suffices for the staining of 12-15 successive strips. Excess of dye is quantitatively removed from the paper by passing it through a battery of five to six baths containing 4% phenol in 10% acetic acd . The electrophoretograms are then dried at 95-100° for 5-10 minutes.

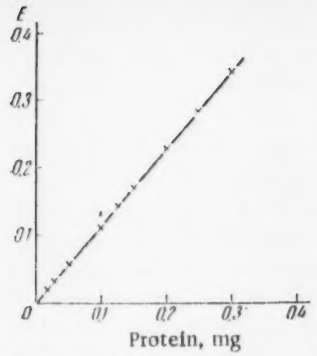

Fig. 2. Dependence of the optical density of dye extracts on the concentration of protein applied to the paper.

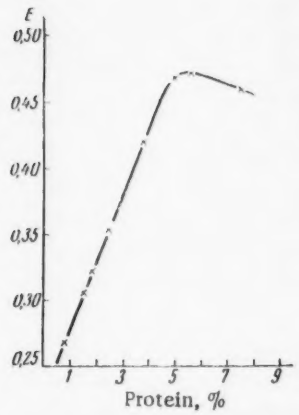

Fig. 3. Curve relating optical density of dye extracts to protein concentration, when high concentrations of protein are applied to the paper.

Determination of the amount of each protein fraction, based on the proportionality between the amount of protein and the amount of dye bound by it, was effected by reading the optical density of extracts of the bands in 0.1 N NaOH. The optical density of the dye extracts was measured (as extinction) in a photocolorimeter, using a green filter (absorption maximum at 520 mμ), zeroing against an extract of unstained piece of paper. The percentage content of the individual protein fractions was calculated taking the sum of the extinctions for all fractions as 100%.

Dependence of the Optical Density of the Dye Extracts on the Amount of Protein Binding the Dye

In order to ascertain the proportionality between the amount of dye bound and the amount of protein present, we prepared a series of serial dilutions of blood serum in physiological saline, and applied 0.1 ml portions of each dilution to paper strips. The protein content of the samples was determined by a micro-Kjeldahl method. The extinctions found were plotted against protein contents of the streaks applied to the paper, expressed as mg. The curve obtained (Fig. 2) shows that the optical density of the dye extracts bears a linear relationship to the amount of protein present, within the range examined (25-250 mg-%).

With more concentrated protein solutions the linear relationship between amount of protein and optical density of dye extract was found to hold only up to protein concentrations less than 5%. When the solution applied to the paper contained more than 5% of protein the amount of dye bound by the protein fell progressively (Fig. 3).

This effect may be ascribed to the formation on the paper of dense masses of colloid, which are not fully stained by the dye. The presence of unstained patches has often been observed in electrophoretic practice, whatever the stain used, usually at the places where protein concentration is highest (in the albumin band). In order to avoid this effect the amount of serum applied to the paper should not exceed 0.015-0.020 ml, or else the serum should previously be diluted with buffer solution.

The proportionality between amount of protein present on the paper and the amount of dye bound by it is also found to hold under conditions of electrophoretic separation of serum proteins into fractions. A comparison of the optical density (as extinction) of dye extracts from spots of whole serum (0.15 ml) with the sum of the densities given by the individual electrophoretic fractions gave the following results:

Whole serum	Electrophoretic fractions
1.06	1.10
1.30	1.24
1.00	0.96
1.06	1.01
1.10	1.03
1.12	1.16

These results show that the amount of dye bound by a given amount of protein was practically the same for the unseparated serum proteins as for its electrophoretic fractions.

It has been reported in the literature that the affinities of serum albumin and globulins for dyes used in

staining electrophoretograms are different. Cremer and Tiselius [5] found that albumin binds bromthymol blue more actively than do the globulins, and these authors suggested that the figures found for the contents of the globulin fractions should be multiplied by a factor of 1.6. Kōiw et al. [6] have drawn attention to the unequal binding capacity for dyes of the various globulin fractions, and they proposed the application of the factors 2.8, 1.7, 1.6 and 1.4 for α_1-, α_2-, β- and γ-globulins, respectively.

We have investigated the binding capacity of albumin and globulins for acid chrome dark blue. Equal volumes (0.1 ml) of a 1.5% solution of globulin* and of a commercial preparation of ovalbumin were applied to paper strips. The globulin preparation was shown to be practically electrophoretically homogeneous. The albumin preparation was found to contain small amounts of two globulin fractions. The spots applied to the paper were dried, stained and extracted, applying the above described procedures. Photometric measurement of the optical density of the extracts showed that albumin bound the dye 1.4 times more actively than did globulin. However, as will be shown below, it is possible to dispense with the application of additional factors for the calculation of the relative globulin contents.

Absorption of protein by the paper. Migration of protein molecules along the paper strips during electrophoresis is associated with absorption of a certain amount of protein by the paper. This absorbed protein takes up dye during staining of the electrophoretogram, and retains it during the washing procedure. The amount of absorption depends on the properties of a given paper, and clearly does not depend on the protein concentration of the solution applied to it. During the electrophoretic separation of different amounts of protein the intensity of staining of the "absorption zone" remains constant, whereas the amount of dye bound, and the intensity of staining of the bands due to the various fractions would fall parallel with the diminution in the amount of protein applied to the paper. If this amount is very small, the bands may not appear at all, since it is all absorbed while traveling along the strip. We found that the absorptive capacity of the paper for albumin is somewhat greater than for globulin. Our experiments showed that an average of 20-30% of the albumin present in serum is absorbed by the paper, under the conditions of electrophoresis applied by us, in which the albumin band moves a distance of 7-8 cm from the starting point.

The globulin fractions, which move about 2-4 cm from the starting point, behind the albumin, are located on the parts of the strip which has absorbed albumin. After staining and extraction of the bands corresponding with the individual fractions the relative values found for the globulin fractions are not only not diminished, but are in fact raised, due to the presence of absorbed albumin in the paper.

As was shown by an examination of electrophoretograms of donor blood-serum proteins, loss of albumins due to their absorption by the paper, and raising of the apparent globulin contents due to absorbed albumin, cancel out each other, because of the higher coefficient of binding of the dye by albumin. In this way, regardless of the more active binding of acid chrome dark blue (and similarly of bromphenol blue) by albumin, as compared with globulins, this difference may be ignored in calculating the relative contents of the various fractions.

Stability of coloration of alkaline extracts of acid chrome dark blue. In order to evaluate the stability of the coloration of dye solutions extracted by 0.1 N NaOH from the various bands of the electrophoretograms, readings of the optical densities were repeated 30 minutes, one hour, and 20 hours after the first reading, taken immediately after extraction.

Table 1 gives the results of such readings, expressed as percentages. We took the optical density of the extracts immediately after extraction as 100%.

We found that a slight fall in optical density of the extracts took place within 30 minutes of extraction.

However, the fall in optical density of the dye extracts during 30-60 minutes was about the same for all fractions, i.e. for different dye concentrations, and was without practical effect on the quantitative results. With further standing (after 20 hours), the greatest percentage fall in optical density was found in the extracts having the lowest dye concentration (the α_1-, α_2- and β-globulin fractions). The density of the dye extracts should, therefore, be read within 60 minutes of preparation.

Reproducibility of the results of staining of electrophoretograms. In order to assess the reproducibility of of staining of the electrophoretograms we determined the contents of serum-protein fractions in parallel experiments. The electrophoretograms were prepared and stained simultaneously, under identical conditions, and using the same amounts of serum. The results are presented in Table 2.

*The globulin preparation was obtained from V.P. Moiseeva, of the Leningrad Blood Transfusion Institute.

TABLE 1

Changes in Optical Density of Dye Extracts on Standing (as percentages of the first readings)

Time of reading	Albumin	Globulins			
		α_1	α_2	β	γ
First reading	100	100	100	100	100
After 30 minutes	93.2	91.1	89.6	89.3	91.8
After 1 hour	92.4	89.6	88.2	88.0	90.8
After 20 hours	57.8	51.4	49.0	46.4	52.8

TABLE 2

Protein Fractions of Blood Serum, as Found from the Analysis of Parallel Electrophoretograms

No. of serum	Relative percentage contents of					No. of serum	Relative percentage contents of				
	albumin	globulin					albumin	globulin			
		α_1	α_2	β	γ			α_1	α_2	β	γ
1	58,9	3,9	6,6	3,5	27,1	6	68,7	7,5	6,5	6,2	11,2
	57,5	3,0	5,7	4,9	28,9		67,7	6,2	7,2	7,5	11,4
2	50,7	4,2	6,0	7,0	32,1	7	62,0	4,9	9,4	9,1	14,6
	48,7	4,4	7,5	7,0	32,4		64,7	4,1	8,8	8,2	14,2
3	65,8	5,0	8,5	10,1	10,6	8	38,3	5,9	9,2	13,5	33,0
	66,5	3,4	10,8	8,8	10,5		37,1	6,3	9,6	13,6	33,4
4	64,8	7,3	7,0	8,9	12,0		37,1	7,1	10,9	13,8	31,1
	64,9	7,2	7,7	8,2	12,0		36,7	7,3	10,3	14,4	31,3
5	57,2	7,2	10,5	14,6	10,5		37,0	5,5	11,0	13,2	33,3
	56,7	7,8	9,9	15,7	9,9		37,2	5,7	10,5	13,2	33,4

It is evident from the table that only small differences are found for the contents of the various protein fractions, as derived from parallel electrophoretograms. When such electrophoretograms are stained in the same bath we found that the absolute values for optical density of the dye extracts were very close to each other, as were also the relative proportions of the individual protein fractions. Considerable differences between the absolute values of the extinction coefficients of the extracts were, however, encountered when parallel electrophoretograms were stained in different baths. The reason for this effect is that the intensity of staining of the electrophoretograms depends not only on the concentration of protein on the paper, and on the duration of the staining process, but also on the time elapsed since the dye solution was prepared. Hence, the intensity of staining of parallel electrophoretograms in different dye baths may vary. This is, however, without effect on the results of calculation of the proportions of the individual fractions present, since the relative intensity of staining of the various bands of a given electrophoretogram remains constant.

SUMMARY

The dye acid chrome dark blue gives rapid and distinct staining of protein fractions in paper electrophoretograms. The stained electrophoretograms do not fade after prolonged storage. An 0.08% solution of the dye in 10% acetic acid is used for the staining bath. The electrophoretograms are stained by immersion in the bath for 12-15 minutes. Excess of dye is removed by passing the papers through a battery of five or six baths containing 4% phenol in 10% acetic acid.

The protein content of the various bands is measured by extracting them with 0.1 N NaOH, and measuring the optical density of the extracts in a photocolorimeter, using a green filter (absorption maximum at 520 mμ).

The amounts of dye extracted from the paper were found to be closely correlated with its protein content. The stability of coloration of the alkaline extracts was investigated, as well as the reproducibility of the staining procedure.

LITERATURE CITED

[1] A.E. Gurvich, Lab. Delo No. 3, 3 (1955).

[2] N.A. Kravchenko, O.P. Samarina and M.G. Kritsman, Biokhimiya 18, 34 (1953).

[3] N. Lazarov, Lab. Delo No. 3, 9 (1955).

[4] F.V. Flynn and P. Mayo, Lancet No. 2, 235 (1951).

[5] H.D. Cremer and A. Tiselius, Biochem. 320, 273 (1950).

[6] E. Kõiw, G. Wallenius and A. Grõnwall, Bull. Soc. chim. biol. 33, 1940 (1951).

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ELECTROPHORETIC STUDY OF THE PROTEIN FRACTIONS OF AZOTOBACTER, IN RELATION TO SPECIES, AGE OF CULTURE, AND SOURCE OF NITROGEN NUTRITION

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Convincing evidence has been forthcoming of recent years that the chemical fractionation methods applied earlier to the study of protein fractions of microbial cells are of limited value only. The application of electrophoretic methods has shown that individual fractions prepared by extraction with various solvents are not, in fact, homogeneous. On the other hand, numerous papers have been published [1-10], in which the authors have shown, by the use of various modifications of electrophoretic methods, that the protein fractions of bacterial cells are highly heterogeneous. It has been demonstrated that electrophoretically different protein fractions may show very considerable quantitative variations, depending on the species [3, 8], the age of the culture [5], and the conditions of cultivation of bacteria [7, 8].

It was necessary, before proceeding to the study of the species specificity of proteins and protein complexes, to ascertain whether the relative proportions of the protein components, and their qualitative properties, very during the process of growth of the microorganisms, and whether they depend on the conditions under which they are cultured. We showed in an earlier communication [9] that the amino acid composition of the "total" protein of Az. agile 22-D undergoes considerable changes during the growth of a culture, but does not depend on the nature of the nitrogenous components of the culture medium used. These changes in the amino acid composition of the "total" protein during the life cycle of the organism might be due either to changes in the amino acid composition of the proteins, taking place during the growth of the culture, or to changes in the relative proportions of the individual component proteins, the composition of each of which remains constant. The possibility cannot be excluded that modification of the structure of the proteins takes place during the process of growth of the cultures. It hence follows that it is essential, for the investigation of the species specificity of bacterial proteins, to examine cultures which are in an identical developmental stage. In our investigation of the amino acid composition of "total" protein we were unable to find any significant differences between the proteins of Az. agile, Az. vinelandii, and Az. chroococcum, examined in the stationary phase of development [10]. This result may indicate that the examination of the amino acid composition of "total" protein cannot provide the necessary evidence for the settling of the question of the existence or absence of species specificity, in many cases, and that it is necessary in such cases to make as full as possible a separation of the protein fractions, and to examine the properties and composition of each fraction separately. In the present paper we give the results obtained from fractionation of the "total" protein of Azotobacter into separate protein fractions, by means of starch gel electrophoresis.

MATERIALS AND METHODS

Azotobacter proteins were examined for different species, ages of culture, and sources of nitrogen nutrition. For the study of the effect of age of culture we took nitrogen-fixing cultures of Az. agile 22-D, at different developmental stages: three-day cultures used for inoculation, cultures in the ten-hour latent phase, in the 28-hour logarithmic phase, and in the 48-hour stationary phase of growth. For the elucidation of the effect of varying the nitrogen nutrition on the nature and amount of the individual proteins of Az. agile 22-D we examined 48-hour cultures supplied with molecular nitrogen (nitrogen-fixing cultures), and 45-hour cultures to which

ammonia had been added (cultures in which fixation of nitrogen had been suppressed). Both cultures were in the stationary phase of growth. Species differences were investigated for the proteins of Az. agile, Az. vinelandii, and Az. chroococcum (Strain 54). All three species of Azotobacter were from a nitrogen-free 48-hour culture, corresponding with the stationary phase of growth. The composition of the medium and the culture conditions were as described in an earlier paper [22].

We used either a freshly prepared bacterial mass, or freeze-dried material. The bacteria were grown in reactors at the Moscow Bacterial Fertilizers Factory.*

It was necessary for the electrophoretic analysis of the bacterial proteins that all the proteins and protein complexes should be extracted as completely as possible, with the least possible denaturation. Attempts to lyse Azotobacter cells with lysozyme were unsuccessful [11]. The use of aluminum dust for the destruction of the cells [12] gave good results, but was associated with firm adsorption of a considerable part of the proteins on the aluminum. The use of sonic vibrations for the destruction of the cells [13] carried the risk of a certain degree of depolymerization of macromolecular components. The procedure applied by us consisted of thorough grinding of the bacterial mass with glass powder, with cooling. In order to ascertain whether the proteins suffered any enzymatic attack during the process of their liberation, we investigated the amount of autolysis taking place in Azotobacter suspensions during 24 hours at room temperature [14]. The results were consistent with the almost complete absence of proteolysis; this is in accordance with published reports of the low protease and deaminase activities of Azotobacter [15].

In order to bring the greatest number of protein complexes into solution we extracted successively with the solvent solutions: 0.14 M NaCl, 1 M NaCl, NaOH solution of pH 8-9, and 0.2% NaOH. Extraction of the protein complexes of Azotobacter with water gave a protein mixture which did not differ in its electrophoretic properties from that obtained by extraction with 0.14 M NaCl. Extraction of each fraction was continued until nothing more was extracted from the bacterial mass by the given solvent solution. The residue from extraction was separated by high-speed centrifugation. All the operations associated with extraction of proteins were carried out at low temperature. Precipitation of protein from the bacterial extracts was effected by addition of neutral ethanol to the salt-solution extracts, and of ethanol made acid to Congo red to the NaOH extracts. The protein precipitating was rapidly collected on the centrifuge, redissolved in a small volume of the same solvent as had been used for extracting it, and the solution was dialyzed against water. The salt-free solutions were then freeze-dried, and the yields of the various fractions were determined.

The supernatant from precipitation of proteins was concentrated in vacuum to about a twentieth of its original volume, and the protein content was determined by the biuret method and by Lowry's method [16]. In no case was any protein precipitated from the supernatant by addition of alcohol.

Analysis of fractions taken for electrophoresis. The bacterial mass taken for fractionation, and the separate fractions obtained, were analyzed for total nitrogen content, nonprotein nitrogen, soluble in 5% trichloroacetic acid (only for the bacterial mass), and purine nitrogen, by Vendrely's method [17]. Nitrogen was determined by a micro-Kjeldahl method. The nucleic acid contents were calculated from the purine nitrogen values, by multiplying by a factor of 8.56; nucleic acid nitrogen was derived by dividing the nucleic acid content by a factor of 5.97, as found in our previous research [18]. Protein nitrogen was calculated as the difference between total nitrogen and the sum of nucleic acid nitrogen and acid-soluble nitrogen (for the bacterial mass only). The protein contents were derived by multiplying the protein nitrogen by the factor 6.25.

The yields of the various fractions taken for electrophoresis are given in Table 1, as well as their protein contents. Analysis of these fractions showed that the salt-solution extracts contained, in addition to proteins, considerable amounts of nucleic acids and polysaccharides. Our procedure of successive extraction of the bacterial mass with a series of different solvent solutions permitted the isolation of about 80-90% of the "total" protein of the bacteria in a soluble form. The insoluble residue, designated by us as "insoluble" protein, represents the difference between "total" nitrogen and that of the sum of the fractions; it amounts to 8-12% for Az. agile and Az. vinelandii, and 20% for Az. chroococcum (Table 3).

Electrophoresis procedure. We dissolved 100 mg of each fraction in 2 ml of veronal buffer, for the electrophoretic separation of its proteins. If the solutions were turbid we clarified them by centrifugation. In most cases

*We are indebted to A.V. Petrov, director of the Factory, and to A.A. Emel'yanova, Head of its experimental laboratory, for their collaboration and assistance in carrying out this part of the research.

TABLE 1

Yields of Fractions Taken for Electrophoresis, and Their Protein Contents (yields are expressed as % of the bacterial mass; proteins as % of the respective fractions)

Growth phase of culture	Nitrogen source	Solvent							
		0.14 M NaCl		1 M NaCl		NaOH, pH 8-9		0.2% NaOH	
		yield of fraction	protein content	yield of fraction	protein content	yield of fraction	protein content	yield of fraction	protein content
Azotobacter agile 22-D									
Inoculation culture	N_2	16.6	14.60	17.2	20.40	25.7	73.23	8.7	82.00
Latent	N_2	30.3	49.00	12.0	62.62	14.7	65.85	0	0
Logarithmic	N_2	26.1	22.31	19.6	24.00	18.4	68.75	6.1	80.37
Stationary	N_2	19.9	18.12	14.5	29.00	23.1	72.50	8.8	83.15
"	NH_4^+	13.6	21.88	24.0	35.44	25.4	58.38	7.1	65.62
Azotobacter vinelandii									
"	N_2	28.7	45.02	9.3	55.48	26.4	54.77	6.7	72.24
Azotobacter chroococcum									
"	N_2	14.8	26.70	12.5	19.38	15.0	46.56	2.9	58.79

TABLE 2

Amino Acid Composition of Protein Fractions (amino acid nitrogen is expressed as percent of total protein nitrogen of the given fraction)

Protein fraction	Solvent solution used for extracting protein mixture	Growth phase	Amino acid												
			lysine	histidine	arginine	aspartic acid	threonine	tyrosine	phenylalanine	alanine	valine	glutamic acid	leucine (isoleucine)	serine	glycine
4*	0.14 M NaCl	Stationary	3,03	2,34	8,82	3,32	2,18	1,40	6,06	7,08	10,53	7,29	14,08	2,80	4,30
4*	1 M NaCl	"	3,02	2,22	8,55	3,30	2,09	1,34	5,90	7,29	10,37	8,01	14,35	2,63	4,58
4*	NaOH, pH 8-9	"	3,25	1,98	9,00	3,51	2,04	1,38	6,15	7,00	10,26	7,82	14,27	2,71	4,63
2*	Total extract	"	5,61	3,71	9,15	5,43	3,32	1,76	2,24	5,78	11,36	12,10	11,36	2,14	4,86
2**	"	Latent	6,16	3,90	10,15	4,98	3,40	1,68	2,02	5,65	10,27	11,93	10,75	2,06	5,00
2**	"	Logarithmic	6,01	3,85	9,84	5,18	3,13	1,86	1,95	5,44	11,16	12,63	10,58	1,90	4,72
5*	"	Stationary	4,14	2,34	8,08	4,31	3,17	1,88	3,52	7,10	10,48	12,40	11,90	2,95	4,88
8*	"	"	4,50	2,64	7,48	4,60	2,76	2,01	4,90	5,26	9,00	8,48	14,36	2,37	4,08

*See Fig. 1.

**See Fig. 2.

we obtained vitreous viscous solutions of mixtures of protein complexes, the separation of which by paper electrophoresis would have been difficult. Very effective separation of the protein complexes was achieved by the use of columns filled with starch gel; the high viscosity of the solutions did not, under such conditions, interfere with the separation. With certain bacteria, such as, for example, Micrococcus lysodeikticus, amylase present in the organisms may cause liquefaction of the starch gel. In such cases, agar or pectin gel may be taken instead of starch.

The gel was prepared from potato starch, in a concentration of 7 g per 100 ml of buffer. The properties of different batches of starch vary, and some are unsuitable for electrophoresis. Before use, the starch was washed, first with tap water, and then with distilled water (until the washings no longer give a positive test for protein), and a fraction was taken which was uniform with respect to sedimentation.

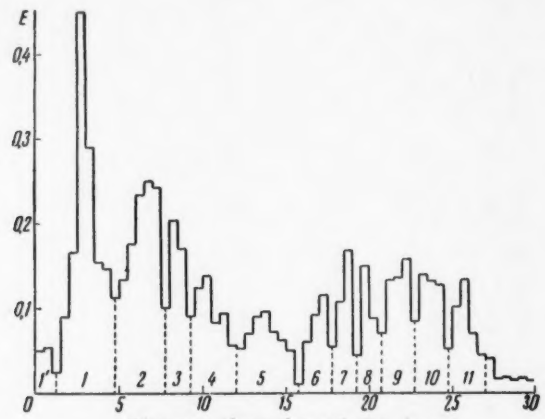

Fig. 1. Electrophoretic separation of a mixture of proteins from Az. agile, in the stationary phase of growth. The fractions are numbered from 1' to 11.

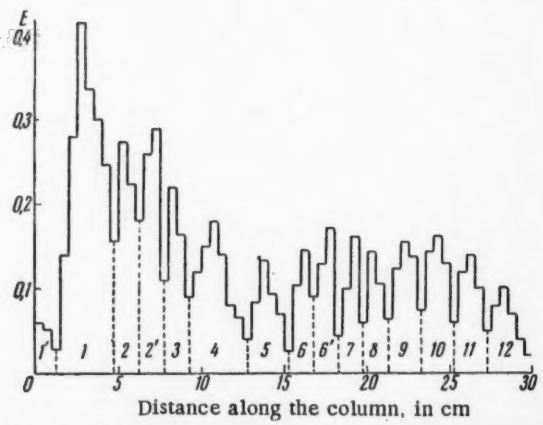

Fig. 2. Electrophoretic separation of a protein mixture from Az. agile, in the stationary (and logarithmic) phase of growth. The protein fractions are numbered 1' to 12.

The optimum voltage and current strength have to be determined for each batch of starch, as well as the concentration and pH of the buffer, and the duration of electrophoresis which ensures the best separation of the protein mixtures under examination. In the present research we used veronal buffer of pH 8.6, ionic strength 0.1 μ.

The duration of electrophoresis was 20 hours, at a voltage of 540 v and a current strength of 50 mA. Platinum electrodes were taken. Water cooling was applied to the columns. We used an instrument designed by Rodionov [19] in our experiments. The working length of the electrophoretic column was 30 cm, and its internal diameter was 3 cm. After completing a run, the contents of the working part of the column were divided into 60 identical segments 0.5 cm in thickness, which were weighed, to an accuracy of ±0.05 g. The weight of the segments varied within the range 3 ± 0.2 g. The dry weight of the starch present in the gel filling the working part of the column was 15 g in all; the average starch content of each segment was thus 0.25 g.

Analytical procedure. Each segment was placed in a thick-walled centrifuge tube, and an equal volume of 4% Na_2CO_3 was added. The gel was cut up into very small fragments with the aid of a lancet, or was ground with a glass pestle, to give a homogeneous suspension. Extraction with the weakly alkaline solution proceeded for 12-15 hours at room temperature, after which an amount of methanol equal to the weight of the segment was added to each tube. The contents of the tubes were well mixed, and were allowed to stand for 2-3 hours, causing coagulation of the starch gel, which became converted into a fibrous mass (average weight 0.5 g), which could be squeezed almost dry. The protein solution was separated from the gel by centrifuging (15 minutes at 3000 rpm), or by squeezing out the fibrous gel. Repeated extraction of the finely dispersed, squeezed-out gel with a small volume of alkaline methanol did not remove any significant amount of protein from it. It may hence be concluded that no appreciable adsorption of protein takes place on the gel.

The protein content of each of the 60 segments was determined by Lowry's method [16]. The optical density of the solutions was measured in an SF-4 spectrophotometer, at a wavelength of 750 mμ, and a light path of 1 cm, and electrophoretic curves were hence constructed (Figs. 1 and 2). The limits of each protein peak were taken as the points of minimum optical density. The sum of the optical densities of each peak is multiplied by the total weight of segments involved, and the product is divided by the product of the sum of all the optical densities and the weight of all the segments; the quotient, multiplied by 100, gives the percentage content of the given protein component in the protein of the fraction subjected to electrophoresis.

Preparative isolation of protein components by the electrophoretic procedure. Basing ourselves on the above-described analytical method for the electrophoretic separation of protein mixtures into their components (Figs. 1 and 2), we proceeded to further experiments, in which we pooled the segments of gel containing a particular protein component, froze the mixture for several hours, and then thawed it out [20]. The fluid contained in the gel, which had a spongy structure after thawing, was separated either by centrifugation at low speeds or by squeezing it out. The residual gel was washed twice with small volumes of buffer solution. The solutions were made weakly acid, and protein was precipitated by addition of alcohol. The amino acid composition of the protein components so prepared was determined by a quantitative paper chromatography method, as described in detail in our earlier papers [10, 21].

EXPERIMENTAL RESULTS

There can be no doubt that most of the protein present in bacterial cells is in the form of complexes with other substances. In conducting an electrophoretic study of bacterial proteins, we can either liberate the proteins from their complexes with these other substances, by chemical or enzymatic reactions, or we can retain the native state of complexing of the proteins with other cell constituents. The former procedure would involve the partial or total disruption of the native complexes, and might cause serious structural damage to the protein component. For this reason, we chose the second line of approach to the problem, as being more likely to give correct results.

Heterogeneity of the protein complexes of Azotobacter. We were able, by means of our electrophoresis procedure, to separate each fraction obtained by extraction with different solvents into twelve protein components (Figs. 1 and 2). In order to elucidate the reason for the qualitative similarity between components of the same electrophoretic mobility, isolated from different solvent fractions, we examined the amino acid composition of four electrophoretic fractions isolated from the protein mixtures extracted from Azotobacter in the stationary growth phase by 0.14 M NaCl, 1 M NaCl, and NaOH solution of pH 8-9. It is evident from Table 2 that the protein components of the same electrophoretic mobility, separated from the protein mixtures present in different extracts, resemble each other closely in their amino acid composition. It may hence be concluded that protein components of the same type are extracted from cells by different solvent solutions. This may be due to the generalized distribution within the cells of certain of the protein complexes isolated, or to different modes of

combination of these complexes with structural elements of the cells. Extraction with 0.2% NaOH alone, so as to achieve maximum extraction of protein complexes from Azotobacter, is not practicable, since the resulting extracts are heavily contaminated with other extractives, which interfere with the clear-cut electrophoretic separation of the protein components. Apart from this, prolonged contact of the bacteria with alkaline solutions causes considerable changes in a number of the properties of the protein complexes originally present within the cells. When the extracts obtained with four different solvent solutions are mixed, and the mixture is subjected to electrophoretic separation, the same general distribution of the components of the mixture is found.

Since most of the bacterial proteins of Azotobacter are present in the cell in the form of complexes with other substances, it is very difficult to compare the properties of electrophoretically separated protein components entering into the composition of the complexes. Complex compounds made up of the same components may differ in the relative proportions of these components, and such quantitative differences may in many cases affect the electrophoretic mobility of the given complex, as a whole.

In order to establish that this is, in fact, the case, we examined the amino acid composition of four protein fractions (2, 4, 5 and 8, Fig. 1, Table 2) of different electrophoretic mobilities, isolated from Azotobacter in the stationary growth phase. We found that the amino acid composition of protein components of different electrophoretic mobilities, isolated from organisms in the same growth phase, was not uniform, quantitatively; this is clear evidence of their nonidentity.

We next investigated the amino acid composition of the protein components of equal electrophoretic mobility, obtained from organisms in the latent, logarithmic, and stationary growth phases (Table 2), in order to examine the effect of the growth phase on cell proteins. The results for these three protein components show that they have very similar amino acid compositions, irrespective of the growth phase of the relevant cultures. Differences in the relative proportions of nucleic acids, for example, in nucleoproteins may lead to the same protein component appearing in fractions of different electrophoretic mobility. Our data on the amino acid composition of the proteins of bacteria at different growth phases do not, however, provide any evidence of this happening. Changes in the free nucleic acid content of bacterial cells are encountered in the course of their growth cycle. It may be supposed that with increasing age of the cell the degree of saturation of proteins with nucleic acids changes very little, if at all, and that as a result of this native nucleoproteins possess a well-defined electrophoretic mobility [6]. Fully valid data should therefore result from a study of the variations in the contents of protein components of equal electrophoretic mobility, over the whole of the growth cycle of the organisms.

The proteins of Azotobacter can,at all growth phases,be separated into about twelve electrophoretic fractions, and this suggests that these fractions were present in the intact cells. It is possible that this figure of twelve fractions is not the final one, and that further improvements in the electrophoretic separation method may lead to the further subdivision of any or all of these fractions. However this may be, there can be no doubt of the intracellular heterogeneity of the proteins of Azotobacter, as shown by our experimental results. The application of physicochemical, enzymatic, and immunological methods of research may be expected,in the future, to provide a definitive answer to the question of the heterogeneity of the electrophoretically separated protein components of the cells.

Changes in the protein components of Azotobacter, in relation to the growth cycle. Table 3 gives the content of each protein component, as a percentage of the "total" protein, over the whole of the growth cycle of Az. agile 22-D. The very small variations found in the percentage contents of most of the fractions (3, 4, 5, 7, 8, 9, 10, 11) provide indirect evidence of the similarity of protein complexes having identical electrophoretic mobilities, over the whole of the growth cycle of the bacteria.

On the other hand, the presence of age differences in the percentage content of protein fractions 2 and 6 is evidence of their heterogeneity. Figure 2 shows that these protein fractions have undergone subdivision during the latent and the logarithmic phases (Figs. 1 and 2; subfractions 2', 6' and 12).

The nucleic acid contents of fractions 2', 6' and 12, which make their appearance during the latent and logarithmic phases, were found to be higher than in other fractions; this is in conformity with our earlier finding [22] that the nucleic acid and nucleoprotein contents of Azotobacter cells are highest during these same growth phases.

No definite conclusions can be drawn from certain variations found in the percentage content of fraction 1,

since this fraction contains small amounts of proteins which had suffered denaturation during the extraction procedure, and which, being difficultly soluble, have a low electrophoretic mobility. The nitrogen content of Az. agile 22-D cells in nitrogen-fixing cultures remains at a constant level during the latent growth phase. The absence of nitrogen fixation during this phase, with simultaneous new formation of nucleic acids, leads to the conclusion that protein nitrogen is being used during the latent phase for the synthesis of nucleic acids. The observed fall in the content of a number of amino acids in Az. agile cells [9] in the latent phase of growth, in particular of tryptophan, proline, dicarboxylic acids, alanine, glycine, and serine, may be related to the utilization of some of these amino acids for nucleic acid biosynthesis. It is of considerable interest to follow the quantitative changes in the respective contents of the individual protein fractions of Azotobacter throughout its growth cycle. Table 4 presents such data for each protein component, in μg per billion cells, over the entire life cycle of Az. agile 22-D.

TABLE 3

Contents of Protein Fractions of Azotobacter (as percentages of the total protein content)

Protein fraction	*Azotobacter agile*					*Az. vinelandii*	*Az. chroococcum*
	inoculation culture	latent phase	logarithmic phase	stationary phase		stationary phase	
	using molecular nitrogen			using N_2	using NH_4^+	using molecular nitrogen	
1'	2,9	3,7	3,0	3,0	2,9	2,9	2,6
1	10,3	14,1	15,7	10,5	10,8	9,9	10,1
2	13,5	9,6	8,3	13,1	12,9	13,7	11,3
2'	0	8,7	2,0	0	0	0	0
3	11,4	8,3	8,0	11,3	11,7	12,0	11,4
4	10,6	8,3	9,1	10,9	10,7	10,2	9,6
5	6,5	7,2	7,7	6,8	7,0	7,3	6,8
6	4,7	5,1	5,3	5,3	5,4	5,7	5,5
6'	0	4,0	2,0	0	0	0	0
7	5,7	5,3	5,8	5,6	5,5	6,0	5,4
8	5,9	4,8	4,7	5,8	5,8	5,4	5,1
9	4,3	5,6	4,6	4,7	4,9	5,2	4,1
10	4,8	6,4	5,4	5,0	5,2	4,6	4,0
11	4,3	6,4	5,9	5,3	5,4	5,1	4,2
12	0	3,5	4,4	0	0	0	0
"Insoluble" protein	15,1	0	8,2	12,5	11,7	12,1	20,0
"Total" protein*	37,50	31,75	30,62	36,44	35,08	42,54	18,83
"Total" protein**	390,00	365,12	303,14	313,38	333,26	340,32	203,36

*As % of the total weight of bacterial mass.

**As μg per billion bacterial cells.

The considerable fall in the contents of fractions 2, 3 and 4, as compared with the inoculation culture, and the slight fall in the contents of fractions 7 and 8, together with the absence of change or the increase in the contents of the other fractions, may be an indication of the selective utilization of certain proteins during the latent phase of growth. The contents of protein fractions 2, 3 and 4 rose during the stationary phase; the contents of these same fractions fell considerably during the latent phase, suggesting that they may have been expended for nucleic acid synthesis, as well as for transformations of existing proteins and for new formation of proteins. The increases observed in the contents of protein fractions 1, 9, 10, and 11 during the latent phase and the beginning of the logarithmic phase of growth, and the appearance of subfractions 2' and 6' and of fraction 12, show that the period corresponding with the most intensive vital activity of the cells is characterized by the activation of numerous systems, and by synthesis of functionally new components.

Effect on the protein components of Azotobacter of varying the source of nitrogen nutrition. Azotobacter

grows more rapidly in media containing ammonium salts as a source of nitrogen than in nitrogen-fixing cultures, thus leading to higher yields of bacterial mass [22]. No difference is, however, found in the amounts of protein accumulating during corresponding growth phases of cultures with or without added ammonium salts. This is evidence that synthesis of reserve proteins of any kind does not take place in Azotobacter cells, even under favorable conditions; only such proteins of the constitutional type as are essential for the vital processes of the organisms are formed. The results of our electrophoretic studies of the protein complexes of Az. agile 22-D, grown in nitrogen-fixing cultures or with ammonium salts, are in close conformity with this view.

TABLE 4

Contents of Protein Components of Az. agile at Various Stages of Its Growth Cycle

Protein fraction	Growth phase				Protein fraction	Growth phase			
	inoculation culture	latent	logarithmic	stationary		inoculation culture	latent	logarithmic	stationary
1'	1,1	1,2	0,9	1,1	1'	11,4	13,5	8,9	9,5
1	3,9	4,5	4,8	3,8	1	40,2	51,4	47,6	33,0
2	5,0	3,1	2,5	4,8	2	52,5	35,5	25,2	40,9
2'	0	2,7	0,6	0	2'	0	31,5	6,1	0
3	4,2	2,6	2,4	4,1	3	44,2	30,1	24,2	35,4
4	4,0	2,6	2,8	4,0	4	41,6	30,4	27,6	34,2
5	2,4	2,3	2,4	2,5	5	25,5	26,1	23,5	21,3
6	1,8	1,6	1,6	1,9	6	18,4	18,6	16,1	16,4
6'	0	1,3	0,6	0	6'	0	14,6	6,0	0
7	2,1	1,7	1,8	2,0	7	22,3	19,2	17,6	17,4
8	2,2	1,5	1,4	2,1	8	22,9	17,5	14,2	18,2
9	1,6	1,8	1,4	1,7	9	16,8	20,6	14,1	14,8
10	1,8	2,0	1,7	1,8	10	18,7	23,5	16,5	15,6
11	1,6	2,0	1,8	1,9	11	16,5	23,3	17,7	16,5
12	0	1,1	1,3	0	12	0	12,6	13,0	0
"Insoluble" protein	5,7	0	2,6	4,6	"Insoluble" protein	58,9	0	25,3	39,2

*As % of total weight of bacterial mass.

**As μg per billion microbial cells.

It is evident from Tables 3 and 4 that the source of nitrogen (N_2 or NH_4^+) does not significantly affect either the qualitative composition of the protein components or their mutual proportions within the cells. We could find no appreciable quantitative differences between the amino acid compositions of the total protein of Azotobacter grown in media containing different sources of nitrogen nutrition (N_2, NH_4^+, and NO_3^-) [10].

Species specificity of the protein components of Azotobacter. The electrophoretic study of the protein components of Az. agile, Az. vinelandii and Az. chroococcum was performed on cultures which were in the same (stationary) growth phase.

It follows from the data of Table 5 that the electrophoretograms of all three species are very similar, in showing the presence of twelve protein fractions. The contents of corresponding protein complexes show fairly wide variations, whether these contents are calculated per standard number of cells or per unit of bacterial mass. The very small quantitative differences found for certain protein complexes only of Az. agile and Az. vinelandii may be related to the considerable physiological resemblances of these two species, as compared with Az. chroococcum. When, however, we calculate the content of the individual fractions as percentages of total bacterial protein, the differences between these three species of Azotobacter become insignificant (Table 3), which appears to be evidence of the functional equivalence of their corresponding protein complexes.

SUMMARY

Starch gel electrophoresis of Azotobacter protein revealed the presence of twelve protein components, differing in their amino acid composition. The amino acid compositions of corresponding fractions (of equal electrophoretic mobility) differ very little at different stages of the growth cycle of the organisms.

TABLE 5

Contents of Protein Fractions in Three Species of Azotobacter

Protein factor	Species of Azotobacter				Protein factor	Species of Azotobacter			
	Chroococcum	Vinelandii	Agile			Chroococcum	Vinelandii	Agile	
			molecular nitrogen	ammonia nitrogen				molecular nitrogen	ammonia nitrogen
1[1]	0,5	1,2	1,1	1,0	1[1]	5,3	9,8	9,5	9,7
2	1,9	4,2	3,8	3,8	1	20,6	33,7	33,0	36,1
2	2,1	5,8	4,8	4,5	2	22,9	46,6	41,0	43,1
3	2,1	5,1	4,1	4,1	3	23,1	40,7	35,4	39,1
4	1,8	4,3	4,0	3,7	4	19,6	34,7	34,2	35,6
5	1,3	3,1	2,5	2,5	5	13,8	24,9	21,3	23,4
6	1,0	2,4	1,9	1,9 *	6	11,1	19,3	16,4	17,9 **
7	1,0	2,5	2,0	1,9	7	10,9	20,4	17,4	18,2
8	1,0	2,3	2,1	2,0	8	10,4	18,6	18,2	19,5
9	0,8	2,2	1,7	1,7	9	8,3	17,6	14,8	16,4
10	0,8	2,0	1,8	1,8	10	8,1	15,7	15,6	13,3
11	0,8	2,2	1,9	1,9	11	8,5	17,3	16,5	17,9
"Insoluble" protein	3,8	5,2	4,6	4,1	"Insoluble" protein	40,7	41,3	32,2	39,0

*As % of total weight of bacterial mass.

**As μg per billion microbial cells.

Very considerable quantitative differences in the contents of the individual protein components of the cells are found in different phases of the growth cycle of Azotobacter, and in certain of these phases qualitatively different protein complexes make their appearance.

An abrupt fall in the contents of certain protein fractions of Azotobacter was found in the latent growth phase, together with a rise in the nucleic acid content; this may be evidence of the utilization of some of the amino acids of these proteins for the biosynthesis of nucleic acids. Varying the source of nitrogen nutrition of the organisms did not bring about any appreciable differences in the nature of the protein components or in their relative contents within the cell.

The same set of protein fractions was found in Az. agile, Az. vinelandii, and Az. chroococcum, although wide variations in the contents of individual fractions were found for different species: Az. agile and Az. vinelandii differed sharply from Az. chroococcum in this respect.

In conclusion, we take pleasure in expressing our gratitude to Candidates of Biological Sciences V.M. Rodionov and V.D. Uspenskaya, and to Junior Scientific Assistant O.G. Zamyatkina, for their assistance in carrying our this research.

LITERATURE CITED

[1] N.J. Berridge and C.A. Briggs, Nature 173, 486 (1954).

[2] E.L. Hess and H.D. Slade, Biochim. et biophys. acta 16, 346 (1955).

[3] E.L. Hess, H.D. Slade and J. Kamine, J. Bacteriol. 74, 661 (1957).

[4] J.M. Rhodes, Dansk tidsskr. farmaci 2, 246 (1956).

[5] T. Berti, Giorn. microbiol. 1, 316 (1955-56).

[6] A.B. Pardee, K. Paigen and L.S. Prestidge, Biochim. et biophys. acta 23, 162 (1957).

[7] M.M. Vorob'eva, Doklady Akad. Nauk SSSR 115, 385 (1957).*

[8] J. Wagman, E. Pollack and E.J. Weneck, Arch. Biochem. and Biophys. 73, 161 (1958).

*See English translation.

[9] G.N. Zaitseva and A.N. Belozerskii, Mikrobiologiya 26, 533 (1957).•

[10] A.N. Belozerskii, G.N. Zaitseva and N.P. Tyuleneva, Mikrobiologiya 27, 7 (1958).•

[11] R. Repaske, Biochim. et biophys. acta 22, 189 (1956).

[12] H.J. McIlwain, Gen. Microbiol. 2, 288 (1948).

[13] G.M. Allen and E.H. Cota-Robles, J. Bacteriol. 74, 79 (1957).

[14] A.N. Belozerskii and N.I. Proskuryakov, Practical Manual of Plant Biochemistry [in Russian] (Soviet Science, Moscow, 1951).

[15] H.L. Jensen, Bacteriol. Reviews 18, 195 (1954); M.V. Fedorov, Biological Fixation of Atmospheric Nitrogen [in Russian] (Sel'khozgiz, Moscow, 1952) p. 88.

[16] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193, 265 (1951).

[17] R. Vendrely, Biochim. et biophys. acta 1, 95 (1947).

[18] G.N. Zaitseva and A.N. Belozerskii, Mikrobiologiya 26, 722 (1957).•

[19] V.M. Rodionov, Zh.A. Antakol'skaya and L.A. Loboda, Labor. Delo (1959) [in press].

[20] O. Smithies, Biochem. J. 61, 629 (1955).

[21] G.N. Zaitseva and N.P. Tyuleneva, Labor. Delo No. 3, 24 (1958).

[22] A.N. Belozerskii, G.N. Zaitseva, L.P. Gavrilova and L.A. Mineeva, Mikrobiologiya 26, 409 (1957).•

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•See English translation.

ISOLATION AND EXAMINATION OF PURE ANTIBODIES, PREPARED BY THE USE OF ANTIGENS FIXED ON CELLULOSE

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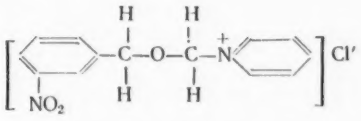
It is of great importance, for the solution of many of the problems of immunochemistry, to have the possibility of isolating pure antibodies from immune sera. A number of procedures for the purification of antibodies are now in use [1]. Most of these procedures, however, serve for the preparation of antibodies in a more concentrated form, rather than for their isolation in a pure state. Of the known methods for the isolation of pure antibodies, the most important is that based on partial digestion of toxin-antitoxin complex precipitates [2]. Only a small number of antitoxins can, however, be prepared by this method. A more universally applicable method for the isolation of pure antibodies could be that based on the use of antigens bound to cellulose [3, 4] or to ion exchange resins [5]. Such methods do not, however, appear to have been practiced at all widely. Apart from this, the authors of the papers cited confined themselves to a description of the method, without giving the results of an examination of the properties of the antibodies isolated.

In the present paper we describe our attempts at the isolation of pure antibodies, by a method reported earlier by one of us [6], depending on chemical fixation of antigen on cellulose, and some of the properties of the antibodies so prepared are given. Our method was based on our still earlier work on precipitation of antibodies on paper [7, 8]. The present paper describes the further continuation of research work, described in a preliminary communication [9].

METHODS

For the fixation of protein antigens to cellulose we used the pyridinium salt of the following structure, which reacts energetically with cellulose:



(1)

The use of this compound for other purposes, and its synthesis, have been described by Kursanov and Solodkov [10].

Preparation of cellulose powder. Cellulose powder was prepared from linter by treating it with boiling alcohol containing 7% of acetyl chloride, for one hour [11].

Preparation of the sorbent (fixation of antigens on cellulose powder). The process of fixation of protein antigens on cellulose resembles that described earlier for paper discs [6]. A weighed amount (50 g) of cellulose powder is treated with 100 ml of a 2% (or 7%) solution of pyridinium salt in 0.7% (or 2.5%) sodium acetate solution, the suspension is mixed, the product is dried at 60-80°, and then heated at 125° for 40 minutes; under these conditions the residue of the salt (1) enters into firm chemical combination with cellulose. The product is washed on a Buchner funnel with tap water, for 20 minutes, and then dried at 80°. The dry powder is washed

with three 200-ml portions of benzene, dried on a Buchner funnel, transferred to a beaker, and treated with 300 ml of 15% sodium hydrosulfite solution at 50-60° (30 minutes, with constant stirring). The product is collected on a Buchner funnel and washed with tap water for 20 minutes. During treatment with hydrosulfite the nitro group of salt (1) is reduced to an amino group. The powder is washed with three 200-ml portions of 30% acetic acid, then with water for 20 minutes, and transferred to a beaker containing cooled diazotizing mixture (1.5% $NaNO_2$ in 5% HCl). The mixture is stirred for 30 minutes and then filtered, and the residue is washed with tap water for five minutes and then dried. During diazotization, the amino group of the pyridinium complex is converted into diazonium salt. For coupling with protein, the partly dried powder is placed in a conical flask containing 150 ml of protein solution (10, 20 or 25 mg per ml) in borate buffer of pH 8.6 and the mixture is left in the refrigerator for 24 hours, with constant stirring by means of a magnetic stirrer. The powder is washed thoroughly with water and physiological saline, to remove all traces of uncombined protein from it. Veronal buffer of pH 9.2 containing β-naphthol is added to the powder, and the mixture is left overnight; no residual unreacted diazo groups can be detected after this treatment. The sorbent is air-dried, and is stored in a desiccator over sulfuric acid. It retains its activity for at least a few months.

Filling of the column. The columns were 0.8 cm diameter by 25 cm long, or 1.6 x 28 cm. They were filled with 1% NaCl solution, and a tube 40 cm long, and of the same diameter as the column, was attached to the upper end of the column. We suspended 2-10 g of sorbent in 25 ml of 1% NaCl solution, and introduced part of the suspension into the upper extension of the column. After most of the powder had settled we opened the lower tap of the column, to drain off some of the fluid, and then poured the rest of the suspension into the upper part of the column. After sedimentation of all the powder was completed we disconnected the upper extension, and connected the column through a three-way tap to a separatory funnel fastened at a height of about a meter above the top of the column.

"Saturation" of the sorbent. "Saturation" was achieved in two ways: 1) immune serum diluted with an equal volume of 1% NaCl (10-40 ml) is passed through the column (it is in some cases desirable to recycle the effluent through the column for two hours), or 2) a suspension of sorbent in immune serum is stirred at room temperature for two hours.

Elimination of protein not bound to the sorbent. In order to eliminate unreacted proteins from the sorbent we passed 1% NaCl through the column, or we suspended the sorbent in 1% NaCl in a beaker, with continual stirring, and periodically renewed the wash solution after filtration through a sintered glass funnel. In either case, washing was continued until the protein content of the effluent or filtrate fell below 5-10 μg per ml.

Elution. Elution was in most cases effected by passing 1% NaCl solution of pH 3.2 through the column. In a few cases we used other eluting solutions. The eluent was passed through the column, and the effluent was collected with the aid of an automatic fraction collector. The time taken to collect each fraction varied from 3 to 30 minutes. Appropriate amounts of borate buffer of pH 8.2 are previously placed into each collecting tube, to neutralize the eluate fractions.

Determination of protein content. Protein content was determined by our modification [13] of Lowry's method [12].

Determination of antibody content. The antibody content was determined by a slightly modified Heidelberger method [13, 14].

Concentration of eluates. The eluates were concentrated by evaporation at room temperature, with simultaneous dialysis; this procedure permits the raising of the protein concentration without raising the salt concentration.

Antigens. The antigens used were crystalline horse serum albumin, crystalline hen's egg albumin, and human serum albumin, isolated by precipitation with alcohol.

EXPERIMENTAL RESULTS

Isolation of Pure Antibodies

Formation of complexes of protein antigens fixed on cellulose with the corresponding antibodies. It was first necessary to ascertain whether antigen fixed on cellulose retained its power of combining specifically with

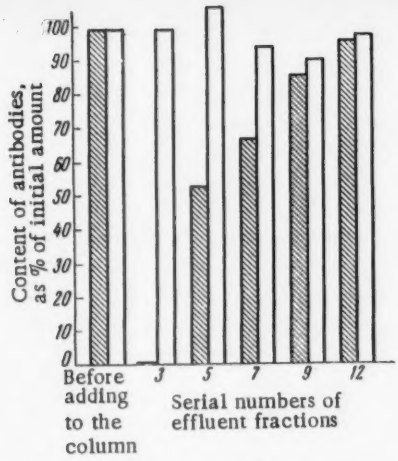


Fig. 1. Content of antibodies to horse serum albumin and to egg albumin in rabbit serum before and after passing it through a column filled with cellulose to which horse serum albumin has been fixed. We passed 60 ml of serum in 1:1 dilution through an 0.8-cm diameter column containing 2 g of sorbent. Five-ml fractions of effluent were collected at 20-minute intervals. The shaded columns represent horse serum albumin antibodies, and the unshaded columns egg-albumin antibodies.

the corresponding antibody. For this purpose, we prepared a column filled with cellulose powder combined with horse serum albumin, and passed through it a specimen of rabbit serum containing two antibodies: against horse serum albumin (2.48 mg/ml), and against egg albumin (3.08 mg/ml). The content of each of these antibodies was determined in the individual fractions of effluent.

Figure 1 presents the results of an experiment of this type. Horse serum albumin antibodies were entirely absent from the first portion of effluent (Fraction 3), whereas the content of egg albumin antibody remained at the initial level. The content of horse serum albumin antibodies rose in successive fractions of effluent, pointing to the gradual "saturation" of the sorbent. At the end of the experiment (fraction 12) the content of horse serum albumin antibodies in the effluent was the same as in the original serum. The sorbent in the column was hence fully saturated at this point, and was unable to bind any more horse serum albumin antibodies. The sorbent did not bind any egg albumin antibodies at any stage of the experiment; the content of these antibodies was about the same in all the effluent fractions, and did not differ significantly from that of the original serum. Similar results were obtained from a number of other experiments. These experiments showed that fixation of the proteins on cellulose did not affect their capacity for forming specific complexes with the appropriate antibodies.

Elimination of serum proteins which have not reacted with fixed antigen. By very quickly filtering 1% NaCl solution through a column, all the proteins, which were not bound to the sorbent, were washed out. After 2-4 hours (depending upon the size of the column) the protein content in the effluent was usually decreased from 2-10 μg to 1 mg, after which the washing was terminated (Table 1).

Obtaining pure antibodies by destruction of the antigen-antibody complexes. It has been shown that the best way to break down antigen-antibody complexes is to treat them with acid solutions [15]. We usually used 1% NaCl solution brought to pH 3.2 by adding 0.1-N HCl, for breaking down the antibody-fixed antigen complexes. It was shown by special control experiments that this solution did not release antigen fixed to the cellulose. When the solution was passed through a column containing "saturated" sorbent a protein appeared in the effluent, which, as will be shown, consisted of antibody. Figure 2 illustrates the results of one such experiment. Protein appeared in the effluent immediately after adding 1% NaCl solution of pH 3.2 to the column containing washed

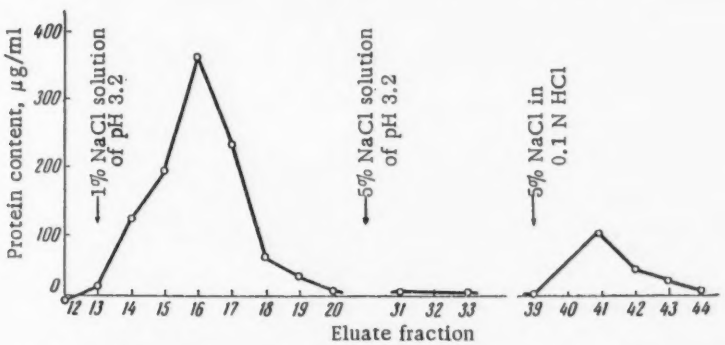


Fig. 2. Elution by various solutions of horse serum albumin antibodies fixed on the sorbent.

TABLE 1

Diminution of Protein Content of Successive Fractions of Effluent During Washing of the Column With 1% NaCl Solution

Serial No. of fraction	Time required for collection of fraction, min	Protein content of effluent, in μg per ml
13	5	23360*
14	10	7660
15	20	190
16	20	17
17	20	18
18	20	6

*Passage of wash-fluid was begun after collecting Fraction 13.

saturated sorbent. Its concentration rose fairly rapidly, to a maximum, and then fell sharply. About half of the antibodies bound to the sorbent were thus eluted by 1% NaCl solution of pH 3.2. We tried to remove the remaining antibodies by raising the ionic strength of the eluent (5% NaCl solution of pH 3.2), and by using more acid eluent (5% NaCl in 0.1 N HCl). No further protein was, however, released from the column by the former solution, while the latter gave only a small additional release of protein. This incomplete elution of fixed antibodies may be due to some of the antibodies forming more tightly bound complexes with antigen than others. We expect that future work will reveal the conditions necessary for the dissociation of such complexes. It is conceivable that some irreversible changes take place during the reaction between antigens and antibodies.

After completing elution, we washed the sorbent, and used it again for further experiments. The capacity of the sorbent for binding antibody fell to about a half after it had been used in an experiment.

An experiment was designed to ascertain whether the eluates contain, apart from antibodies, other serum proteins which might have unspecifically adsorbed on the cellulose powder during the passage through it of rabbit serum. With this object, we passed serum from a nonimmunized rabbit through a column. The column was then washed with 1% NaCl solution, as before. When 1% NaCl solution of pH 3.2 was then passed through the column no protein could be detected in the effluent. It is concluded that the eluate does not contain appreciable amounts of unspecific protein.

Study of Some of the Properties of the Pure Antibodies Isolated

Immunochemical study of the eluted protein (antibody). In order to establish that the protein present in the eluates is actually antibody, we determined the total protein content and the content of precipitating antibodies. For this purpose we used both individual eluate fractions and mixtures of concentrates of a number of fractions. As may be seen from Table 2, practically the whole of the protein present in the eluates was precipitated by antigen, i.e. it was precipitating antibody.

TABLE 2

Content of Precipitating Antibody to Horse Serum Albumin Found in the Eluates

Total protein content of eluate, in μg	Amount of antigen added, μg	Precipitate formed, μg	Antibody content, μg	%
223	25	232	209	93
213	35	263	228	107
2912	300	3300	3000	103
4510	350	3920	3570	79
90	6	46	40	44

In some of the experiments not all of the protein present in the eluate was precipitated, part of it remaining in solution. The curves of Fig. 3 illustrate the changes in the protein content of the solution and the precipitate following addition of various amounts of antigen to the eluate. In this case not more than 45-80% of the total protein present in the eluate could be found in the precipitate. This finding may have been due to the following causes: first, it is known that the precipitate has a certain solubility in physiological saline (from 30

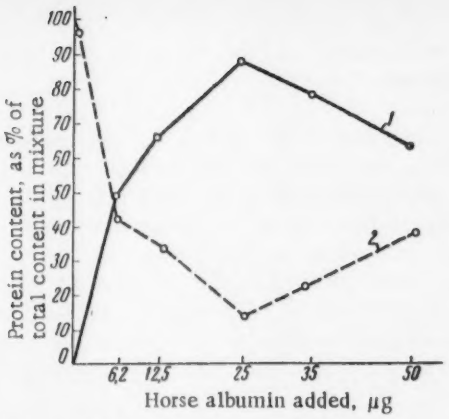


Fig. 3. Changes in the proportions of protein found in the precipitate and in solution following addition of different amounts of antigen to an eluate containing antibody against horse serum albumin: 1) protein content of the precipitate; 2) protein content of the solution.

to 300 μg of precipitate protein per ml) [1]; second, some of the antibody may have undergone inactivation during prolonged storage, and in particular during the process of concentration at room temperature; and, third, the eluate may, in some cases, contain nonprecipitating antibodies.

Immunochemical study of antibodies bound to paper. The ability of the isolated antibodies to react with their respective antigens was examined not only in solutions, but also with antibodies bonded to paper. For these experiments we used an 0.2% solution of antibodies (80% precipitable) against horse serum albumin. Binding to paper was achieved using paper discs 2.0 cm in diameter, treated with a 10% solution of pyridinium salt, by the previously described procedure [6]. Horse serum albumin was applied to such discs (200 μg per disc). Unreacted albumin was washed out of the discs with veronal buffer of pH 9.2, after which the protein content of each disc was determined. In order to determine the extent of unspecific adsorption of protein we applied horse serum albumin to a disc, to which unspecific serum proteins aad previously been bound. As appears from Table 3, the increase in protein content of discs containing fixed antibodies amounted to 11 μg, as compared with 4 μg for discs containing fixed unspecific serum proteins. The specific increase thus amounted to 7 μg. It may be concluded that the isolated antibodies retained their ability to react with antigen after fixation on paper.

Electrophoretic examination of isolated antibodies. The concentrated isolated antibody fractions were subjected to electrophoresis in a Tiselius-Svenson instrument.* The electrophoretograms showed only one peak (Fig. 4), pointing to the absence of contaminating admixtures. The band was, however, somewhat diffuse, and resembled that given by the electrophoresis of the γ-globulin fraction of the serum of nonimmunized rabbits. Comparison of the electrophoretic mobilities of two preparations of antibodies against horse serum albumin, a preparation of antibody to egg albumin, and a preparation of γ-globulins separated by starch slab electrophoresis from the serum of nonimmunized rabbits, showed that there were certain differences in the electrophoretic mobilities of these proteins (Table 4).

TABLE 3

Reaction of Antibody Fixed to Paper Discs With the Corresponding Antigen

Description of the protein bound to the paper	Amount of horse albumin applied, μg	Amount of protein found in disc, μg
Antibody against horse serum albumin	–	25
	200	36
Unspecific serum proteins	–	41
	200	45

The absence of contaminants from our preparations was also demonstrated by the results of paper electrophoresis (Fig. 5).

*The electrophoretic examination of the preparation in the Tiselius instrument was done by V.D. Uspenskaya, to whom we have pleasure in expressing our gratitude.

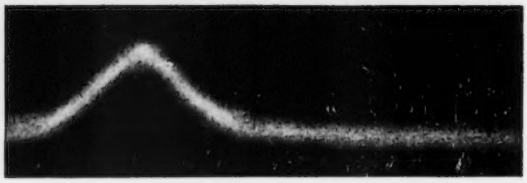


Fig. 4. Electrophoretogram of isolated antibody against horse serum albumin, 2 hours after beginning electrophoresis in a Tiselius-Svenson instrument. Borate buffer, pH 8.2; x = 0.0025.

TABLE 4

Electrophoretic Mobility of Pure Antibodies and of Rabbit Serum γ-Globulin

Description of protein	Electrophoretic mobility, $cm^2\ sec^{-1}\ V^{-1}$
Serum γ-globulins from a nonimmunized rabbit	$1.4 \cdot 10^{-5}$
Antibodies against horse serum albumin	$1.22 \cdot 10^{-5}$
The same (different preparation)	$1.05 \cdot 10^{-5}$
Antibodies against egg albumin	$1.64 \cdot 10^{-5}$

Examination of Sedimentation of Antibodies*

Sedimentation rates were measured in a Svedberg ultracentrifuge; speed of rotor 55,000 rpm, temperature in the rotor 20°. We used a 1% solution of protein in borate buffer of pH 8.2. We compared the sedimentation rates of antibody against horse serum albumin and of the γ-globulin fraction of nonimmunized rabbit serum. The sedimentation diagrams are shown in Fig. 6. The conclusion may be drawn from these preliminary experiments that the eluates examined do not contain any admixture of contaminants, and that the sedimentation constant for isolated antibody is equal to 6.1 Svedberg units.

Content of unspecific admixtures in the precipitate. It is of some importance to ascertain whether appreciable amounts of unspecific serum proteins are trapped in the reaction between antibody and fixed or dissolved antigen, when this reaction proceeds in the presence of serum. Should the antigen-antibody complex be contaminated with any considerable amounts of unspecific proteins, these would unavoidably appear in the eluate obtained when the complex is dissociated. Apart from this, the presence of unspecific admixtures in the precipitate is of importance for the interpretation of the results of quantitative immunochemical analyses [13, 14].

We investigated this problem by conducting the precipitation reaction with concentrates of solutions of pure antibodies, in the presence or absence of nonimmunized rabbit serum (Table 5).

As is evident from Table 5, the precipitate of antibody-antigen complex formed in the presence of heated serum contained 1.7% of unspecific proteins. Higher contents (8.4%) were found when unheated serum was present.

TABLE 5

Content of Unspecific Admixtures in the Precipitate

Taken for the reaction							
antibody solution		antigen solution		unspecific serum		Amount of precipitate formed, μg	Content of unspecific protein in the precipitate, %
volume, ml	amount of protein, μg	volume, ml	amount of protein, μg	heated at 56° for 30 min, ml	unheated, ml		
0.103	465	0.103	26	–	–	356	–
0.103	465	0.103	26	0.103	–	363	1.7
0.103	465	0.103	26	–	0.103	389	8.4

Heterogeneity of Pure Antibodies

We have thus been successful in preparing purified antibodies, which were fully precipitated when the appropriate antigen was added, and in which the presence of impurities could not be detected by electrophoretic

*The sedimentation measurements were made in the Laboratory of Protein Structure, Institute of Biological and Medical Chemistry, for which we are deeply indebted to Prof. V.N. Orekhovich and to Scientific Assistant V.O. Shpikiter.

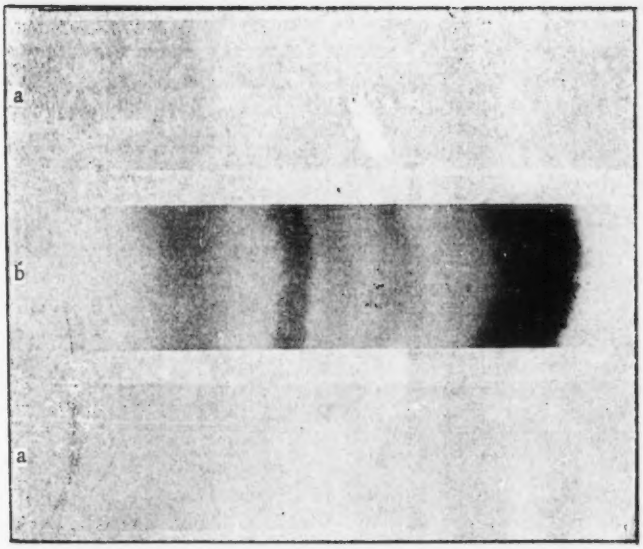


Fig. 5. Paper electrophoretograms of isolated antibodies (a) and of rabbit serum (b). Veronal-medinal buffer, pH 8.6; time 18 hrs.

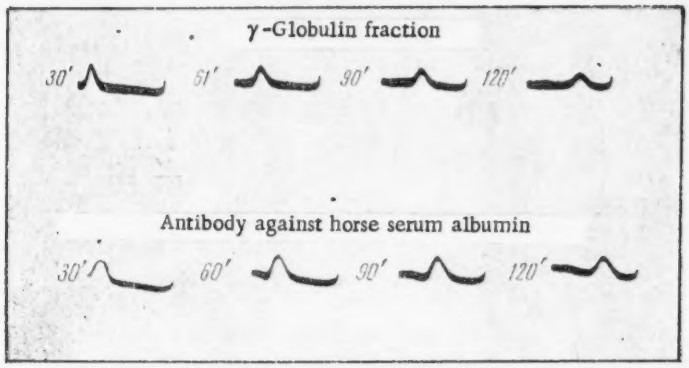


Fig. 6. Sedimentation diagrams given by nonimmunized rabbit serum γ-globulin and by antibody against horse serum albumin. Rotor speed 55,000 rpm; rotor temperature 20°.

or sedimentational methods. Nevertheless, such purified antibodies were found to be heterogeneous, by a number of tests.

Differences between the antibodies present in different eluate fractions, with respect to formation of soluble complexes with excess of antigen. Immunochemical examination of the separate fractions of eluted antibodies against horse serum albumin revealed certain differences in their behavior. The ability of the antibodies to form soluble complexes in the presence of excess of antigen was found to vary from fraction to fraction. As is known, addition to an antibody of excess of a protein antigen leads to the formation of soluble antigen-antibody complex, and a precipitate is not formed. This effect was observed when horse serum albumin was added to the first antibody-containing fractions of eluate (curve obtained for fraction 21, in Fig. 7). Fractions of antibody eluted subsequently gave a precipitate, however, even in the presence of an excess of antigen, and the amount of precipitate formed rose with increase in the serial number of the fraction (curves for fractions 23, 25, and 27, in Fig. 7).

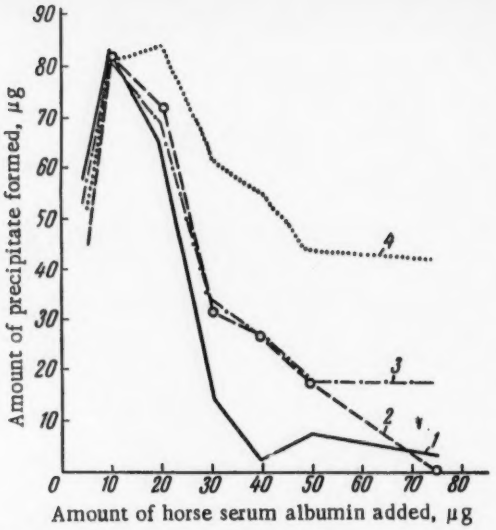


Fig. 7. Dependence of the amount of precipitate on the amount of horse serum albumin added to different fractions of eluted antibody against horse serum albumin: 1) fraction 21; 2) fraction 23; 3) fraction 25; 4) fraction 27.

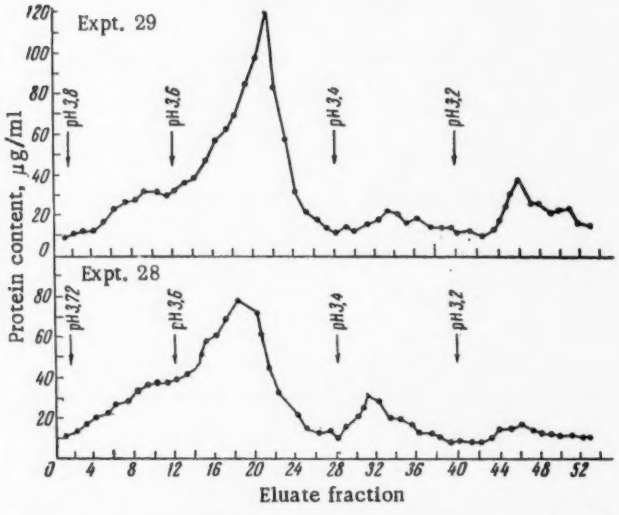


Fig. 8. Elution by means of solutions of different acidity of antibodies against horse serum albumin bound to the sorbent.

It is evident from the figure that the maximum amount of precipitate formed (and hence the total antibody content) was the same for all eluate fractions, but that the amount of precipitate varied in systems containing excess of antigen.

Further evidence of differences between antibodies of different eluate fractions is provided by the observation that, in many experiments, turbidity appeared in certain definite fractions of eluates of antibodies against horse serum albumin (the fraction of the highest protein content, and the immediately succeeding one or two

fractions), followed by separation of a small amount of protein precipitate. All the other fractions remained quite clear, even after prolonged storage in the refrigerator.

Separation of antibodies by elution with solutions of different acidity. An attempt was made to fractionate antibody against horse serum albumin by elution with 1% NaCl solutions of different acidities (pH 5.2, 4.2, 4.0, 3.8, 3.72, 3.4, 3.2). We found that solutions of pH 5.2 and 4.2 did not elute antibody. Antibodies were eluted by more acid solutions (Fig. 8), the bulk of the material being extracted by solutions of pH between 3.8 and 3.6 (74%). Some additional amounts were released from the sorbent by solutions of pH 3.4 and 3.2 (12 and 14%, respectively).

Separation of isolated antibodies into fractions soluble and not soluble in distilled water. A concentrated solution of pure antibodies against horse serum albumin was dialyzed against distilled water for three days, in a radial dialyzer. This treatment separated the antibodies present in the extract into an insoluble fraction (euantibodies, 24.6%), and a soluble fraction (pseudoantibodies, 75.4%).

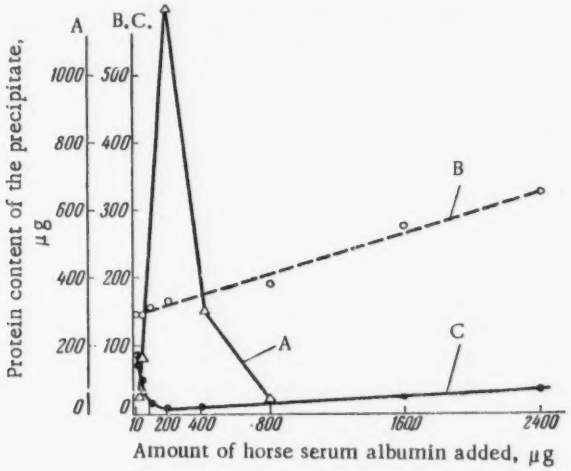


Fig. 9. Relation between the amount of precipitate obtained and the amount of antigen added to sera taken at different times after immunization: A) rabbit serum taken on the ninth day after immunization; B) rabbit serum taken on the fourth day after immunization; C) antibodies isolated from serum B.

Properties of the antibodies present in the immune sera of rabbits on the fourth day after repeated immunization of the animal. The experiments described above were performed on sera taken on the sixth to tenth day after repeated immunization. Inasmuch as these experiments revealed a certain heterogeneity of the antibodies, the possibility existed that this may have been due to the formation at different times after immunization of different antibodies [16]. In order to check this possibility, we examined specimens of serum taken from rabbits on the fourth day after repeated immunization with horse serum albumin. We found, in a number of experiments, that the curve expressing the relation between the amount of antigen added and the amount of precipitate forming in sera taken on the fourth day after immunization was quite different to that found for sera taken on the seventh to tenth day after immunization (Fig. 9B). The difference was in the absence of dissolution of precipitate, over a wide range of excess of antigen. For comparison, an analogous curve (Fig. 9A) is shown, for serum taken on the ninth day after repeated immunization.

Antibodies were isolated from serum taken on the fourth day after administration of antigen. The curve expressing the dependence of the amount of precipitate on the amount of antigen added to isolated antibodies (Fig. 9C) differed from the corresponding curve found for the same antibodies before their isolation from the serum. However, the isolated antibodies retained their ability to form precipitates in the presence of very large excess of antigen.

DISCUSSION OF RESULTS

The techniques described above have enabled us to isolate pure native antibodies, which precipitate quantitatively when mixed with the corresponding antigen, and which do not contain impurities detectable electrophoretically or in the ultracentrifuge. Similarly to Campbell [15], we used antigen fixed on cellulose for the isolation of pure antibodies. Our procedure differed from that of Campbell, however, in that, instead of using p-nitrobenzyl chloride for attachment of antigen to the cellulose, we used the pyridinium salt (1), which reacts much more energetically with cellulose. Attachment of the protein to cellulose is effected through the diazonium groups introduced into the salt residues joined to cellulose. These groups may react with the phenol groups of tyrosine, the imidazole groups of histidine, the ϵ-amino groups of lysine, the sulfhydryl groups of cysteine, the indolyl groups of tryptophan, the guanidino groups of arginine, and the α-amino groups of glycine [17]. Owing to the multiplicity of groups which can react with the diazonium groups, the latter can serve for the attachment of a large variety of different proteins to cellulose.

After fixation to cellulose by this procedure, the proteins retain their immunological properties. Thus, protein antigens attached to cellulose are able to react with the corresponding antibodies, and attached antibodies with antigens. In this way, the treated cellulose acts as a specific sorbent for immune bodies. Such sorbents permit of the selective extraction of the corresponding antibodies from immune sera. It is, conversely, possible by the use of sorbent with attached antibody to achieve selective extraction of any desired protein from a mixture of proteins. Williams and Stone have recently [18] successfully applied a sorbent of this type to the removal of a given protein from its mixtures with other proteins. Their procedure for the preparation of the sorbent appears to us, however, to be more complicated than is the direct attachment of antibodies to cellulose treated with pyridinium salt (1).

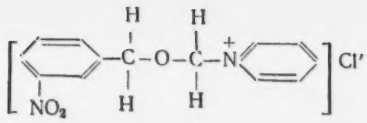
The pure antibodies against serum and egg albumins isolated by us differ from each other, and from unspecific γ-globulins, in their electrophoretic mobility. This finding is in conformity with the independence of the processes of biosynthesis of each of these antibodies, and also of the biosynthesis of unspecific γ-globulins and of antibodies in general, reported by one of us in earlier papers [19, 20].

Antibody against horse serum albumin, which appeared to be homogeneous by immunological, electrophoretic, and sedimentation analysis tests, was found to be heterogeneous in a number of its properties: in the nature of its reaction with excess of antigen, in the stability of its attachment to fixed antigen, and in its solubility in distilled water. Further evidence of heterogeneity was afforded by the shape of the peaks (low peaks on a broad base) observed during electrophoretic or sedimentational analysis of the isolated antibodies.

There may be several explanations of the heterogeneity of the individual antibodies isolated by us. In the first place, a number of different organs of an animal may contribute to production of antibody [21, 22], and each of these organs may produce an antibody differing slightly from those of other organs. Heterogeneity may also result from modifications taking place during the immunization process in the nature of biosynthesis of antibodies by each organ, and also from the formation of a number of types of antibodies against different determining groups of the complex protein antigen molecule. Haurowitz [23] has suggested that the heterogeneity of antibodies may also be related to differences in their degree of "adaptation." Thus, each antibody represents a "family of proteins." Further work on the fractionation of individual antibodies, and on the comparison of the properties of each of the fractions so isolated, is necessary. Of particular interest are the changes taking place in the antibodies present in the circulation at different times after immunization. We have drawn attention to the existence of such changes in an earlier paper [16]. We have also found that the antibodies present in the serum on the fourth day after giving a second injection of antigen differ from those present on subsequent days. Such changes in the properties of antibodies found in the serum on different days after administration of antigen may be due to the different contributions made by different organs to the overall synthesis of antibodies, or to changes in the nature of the biosynthetic process in each of these organs. The possibility of isolating and fractionating pure antibodies should lead to the more profound analysis of this problem.

SUMMARY

A method is described, serving for the attachment of protein to cellulose powder, through the intermediary stage of a compound of cellulose with m-nitrobenzyloxymethylpyridinium chloride:



The attached protein (antibody or antigen) retains its immunological properties. Using antigen so fixed, it is possible quantitatively to remove from immune sera antibodies against any desired antigen, without affecting the content of any other antibodies. The method was applied to the isolation of pure antibodies, by breaking down the antibody – fixed antigen complex, and served for the isolation from rabbit serum of pure antibodies against horse serum, human serum, and hen's egg albumins. The antibodies so isolated retained their biological activity (they were completely precipitated by the appropriate antigens), and were homogeneous by electrophoretic and sedimentation analysis tests.

Differences in electrophoretic mobility were found between different antibodies, and between these and unspecific γ-globulins; the mobility of rabbit antibody to egg albumin was $1.64 \cdot 10^{-5}$, to horse serum albumin $1.2 \cdot 10^{-5}$, and of γ-globulin from nonimmunized rabbit serum $1.4 \cdot 10^{-5}$ cm^2 sec^{-1} V^{-1}.

Antibody to horse serum albumin, although not containing any impurity, was found to be heterogeneous, in a number of respects: in the solubility of its precipitate with excess of antigen, in the firmness of its attachment to fixed antigen, and in its solubility in distilled water.

The pure antibodies were used for the determination of the amount of unspecific serum proteins entrained in the precipitate. It was found that when the reaction between pure antibody and its corresponding antigen took place in the presence of serum from a nonimmunized rabbit heated at 56° for 30 minutes the content of unspecific protein in the precipitate amounted to 1.7%, as compared with 8% when unheated serum was present.

We are deeply indebted to Prof. S.Ya. Kaplanskii for his valuable advice during the prosecution of this research.

LITERATURE CITED

[1] W.C. Boyd, Fundamentals of Immunology (Interscience, New York, 1956).

[2] D. Norton, M. Kunitz and R. Herriot, Crystalline Enzymes [Russian translation] (Izd. IL, Moscow, 1950).

[3] D.H. Campbell, E. Luescher and L.S. Lerman, Proc. Nat. Acad. Sci. USA 37, 575 (1951).

[4] L.S. Lerman, Nature 172, 635 (1953).

[5] H.C. Isliker, Ion-Exchange Resins in Medicine and Biology [Russian translation] (Izd. IL, Moscow, 1956). p. 230.

[6] A.E. Gurvich, Biokhimiya 22, 1028 (1957).*

[7] A.E. Gurvich, Biokhimiya 20, 550 (1955).

[8] A.E. Gurvich and N.G. Karsaevskaya, Biokhimiya 21, 746 (1956).

[9] A.E. Gurvich, R.S. Nezlin and R.B. Kapner, Proc. Annual Conf. Inst. Biol. and Med. Chem [in Russian] (Medical Press, Moscow, 1958).

[10] D.N. Kursanov and P.A. Solodkov, Zhur. Priklad. Khim. 16, 11-12, 351 (1943).

[11] F. Flodin and D.W. Kupke, Biochim. et biophys. acta 21, 368 (1956).

[12] O.H. Lowry, Y.N. Rosebrough, L.A. Farr and R.Y. Randall, J. Biol. Chem. 193, 265 (1951).

[13] A.E. Gurvich and R.B. Kapner, Labor. Delo No. 2, 23 (1958).

[14] E. Kabat and M. Heidelberger, J. Exptl. Med. 66, 229 (1937).

[15] D.H. Campbell and F. Lanni, Amino Acids and Proteins (Springfield, Illinois, USA, 1951) p. 642.

*Original Russian pagination. See C.B. translation.

[16] J.H. Humphrey and R.R. Porter, Biochem. J. 62, 93 (1956).

[17] A.N. Howard and F. Wild, Biochem. J. 65, 651 (1957).

[18] R.R. Williams and S.S. Stone, Arch. Biochem. and Biophys. 71, 377 (1957).

[19] A.E. Gurvich, Voprosy Med. Khim. 1, 169 (1955); Clin. Chim. Acta 1, 101 (1956).

[20] A.E. Gurvich and N.P. Smirnova, Biokhimiya 22, 626 (1957).•

[21] J.H. Humphrey and B.D. Sulitzeanu, Biochem. J. 68, 146 (1958).

[22] B.A. Askonas and J.H. Humphrey, Biochem. J. 68, 252 (1958).

[23] F. Haurowitz, Chemistry and Biology of Proteins [Russian translation] (Izd. IL, Moscow, 1953).

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•Original Russian pagination. See C.B. translation.

THE TRANSFORMATIONS OF NUCLEOTIDES IN NUCLEATED ERYTHROCYTES WITH REFERENCE TO RESPIRATION

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Studies of phosphate metabolism in rabbit erythrocytes undergoing glycolysis and of the respiration of pigeon red blood corpuscles, carried out by Éngel'gardt in 1930-1932 [1-3], showed the existence of a phosphate cycle in the cells which took place with participation of ATP. In the normal course of respiration and glycolysis these cyclic transformations never appeared; the inorganic phosphate (P_I) content and the ATP in the cell did not change. However, if by some process we could cut off the respiration of the pigeon erythrocytes, which were especially suitable for studying the transformations of phosphates in cells with an aerobic type of metabolism, dephosphorylation of ATP began, the content of P_I increased, and thus there appeared a continuous phosphate cycle which did not appear under other conditions. The experiments of particular value for showing the existence of a phosphate cycle and the participation of ATP in cellular respiration were those on pigeon erythrocytes [3] in which, under anaerobic conditions, we first lowered the level of intracellular ATP and then, by saturating the erythrocyte suspension with oxygen, we produced conditions which favored resynthesis. Thereupon we observed esterification of P_I and an increase in the easily hydrolyzed fraction which was basically identical with ATP. These experiments gave direct experimental evidence of the existence of a phase of resynthesis in the reversible phosphate cycle connected with respiration. The data from rabbit erythrocytes also showed the presence in these cells of a phosphate cycle which functioned in an analogous way. However, we showed a difference between the two forms of erythrocytes in that, in the pigeon erythrocytes,resynthesis of ATP was associated with respiration, but in rabbit erythrocytes, with glycolysis. Respiration and glycolysis accounted for the energy expenditure of the phase of resynthesis and assured its overall reversibility. Thus, even in 1932 we had discovered,on a cellular level,the association between phosphorylation and respiration and glycolysis, which several years later was called oxidative phosphorylation.

Further study of the transformations of ATP in connection with cellular respiration showed that under anaerobic conditions it was deaminated [4], but, differing from phosphorus, the ammonia was split irreversibly from the pigeon erythrocytes. These results indicate the deep decomposition of ATP in experimental anaerobic conditions, including its dephosphorylation, deamination, and the accumulation in the erythrocytes of inosine derivatives. The reversibility of the dephosphorylation and the irreversibility of the deamination makes us think that under physiological conditions the transformation of the ATP does not go beyond the limits of the adenylic system [5] and that in the experiments there may be a considerable deviation of erythrocyte metabolism from its normal course. Identification of the products of anaerobic splitting of ATP in pigeon erythrocytes would take on, from this point of view, a very great significance for judging the results of a large number of experiments with resynthesis. The determination of easily hydrolyzed phosphorus,according to Lohmann,which we carried out in earlier work [1, 3],does not permit identification. Later experiments with the determination of ammonia showed only that in anaerobic experiments derivatives of the inosine series accumulate in pigeon erythrocytes but give no means of identifying the products of aerobic resynthesis. Enzymatic determination of ATP with adenyl deaminase [7] also fails to solve the problem, as is now known, because the bicarbonate extract of rabbit muscle does not have sufficient specificity for selective determination of ATP. Later [8] critical analysis of the data from the enzymatic determination showed that it did not exclude formation of ADP under anaerobic conditions and true resynthesis of ATP in pigeon erythrocytes under aerobic conditions.

The uncertainty of the chemical nature of products of anaerobic splitting and aerobic resynthesis of ATP in nucleated erythrocytes gave reason to doubt whether under the experimental conditions there was a true resynthesis of ATP in pigeon erythrocytes [9].

Recent investigations of nucleotide metabolism have shown that in animal tissues there are present almost all the nucleotides of the purine and pyrimidine series and that different kinases exist for transfer of phosphate groups between nucleotides in different directions. This has raised doubts as to whether the binding of inorganic phosphate and the increase in the easily hydrolyzed phosphate fraction in pigeon erythrocytes may not be the result of some side reaction. Oxidative phosphorylation assumes the presence of three processes related to oxidative reactions: binding of inorganic phosphate, esterification of ADP, and formation of ATP. The experiments on pigeon erythrocytes carried out by Éngel'gardt in 1930-1932 could only be considered unreserved experimental evidence for oxidative phosphorylation if it could be shown that all the above processes occurred in them.

In this connection we decided to use present analytical methods to attack the past problems and to repeat the experiments of Éngel'gardt on pigeon erythrocytes [3]. The direct purpose of this work was to identify the products of anaerobic splitting and aerobic resynthesis in the nucleated erythrocytes. Also, setting up experiments on oxidative phosphorylation in intact cells seemed useful to us in other respects. Most of the experiments on oxidative phosphorylation up to now have been carried out on simplified systems, and sometimes on isolated enzyme preparations. Experiments with intact cells could show how much the regularities established in simplified systems exist in vivo. Data related to this idea have been published in part previously [10].

METHODS

For determination of nucleotides, nucleosides, and free bases we used paper chromatography, essentially as was described in previous work [10]. However, because of the complicated analytical problem, we introduced a number of changes and partly used a new method which improved the procedure.

The Paper and Its Preparation

The separation of the nucleotides was carried out on paper GOST 6717.58 of the Leningrad Paper Factory No. 2, and also in part on Whatman No. 1 paper. We used sheets with dimensions of 27 x 33 cm. For the quantitative determination we preferred Whatman No. 1, since it permitted a more rapid, sharp, and reproducible separation. For reproduction of the chromatogram by the contact process it was best to use Leningrad slow paper since the Whatman No. 1 was too thin and its sheets were easily penetrated by ultraviolet rays and the prints reproduced all the details of the paper (especially cloudiness) which makes it difficult to obtain a sufficiently even base. Leningrad slow paper holds back ultraviolet to a greater extent than Whatman, and on light-sensitive paper it has the advantage of causing fluorescence which levels out irregularities of structure which appear more clearly in Leningrad slow paper than in Whatman. It should be remarked that the Leningrad slow paper has a much less standardized structure and thickness of sheets. The increased thickness of sheets causes a lower mobility of substances and so to get the same chromatogram we selected sheets with the same thickness, using a micrometer, and also tried to use sheets which gave more or less the same cloudiness.

For full separation of all the nucleotides of the nucleated erythrocytes we used a two-phase chromatograph, and we changed the method of preparing the paper for this purpose. Washing the paper with versene, which gave very good results with one-phase chromatography, was not sufficient for two-phase chromatograms. Washing with versene, as is known, does not in itself remove impurities from the paper (especially substances which absorb in the ultraviolet) but only prevents the hindering effect of alkaline earth and some of the heavy metals with which versene forms chelates. Organic and some inorganic impurities remain in the paper after it is washed with versene and appear as a dirty front when the chromatographic solvents are passed through. In this respect the flow of solvent is favored if a descending chromatogram is used; then after development of the chromatogram, we can obtain an almost perfectly pure base. The necessary conditions for using such a process of chromatography are that the substances being separated should not pass ahead of the dirty front or coincide with it. These prerequisites are fulfilled in the one-phase chromatograms used in the previous work [10].

Different conditions occur in the two-phase chromatogram. The square sheets of paper used in this case are much shorter than the sheets used for one-phase chromatography, and the time of appearance, and hence the time of flow of the solvent are much decreased. The result of this is that the impurities are not fully removed during passage and accumulate from the beginning at the lower part of the sheet; then when the solvent is passed

in the second direction they shift to the lower left corner and impede the mobility of the fastest moving spots of hypoxanthine and inosine. The unremoved substances which absorb the ultraviolet greatly increase the values of the control in these portions and make impossible the exact quantitative determination of the nucleotides. Therefore, to remove these ultraviolet absorbing impurities from the paper, we washed it with 20-25% formic acid [11]. Washing was carried out for 48 hours by the chromatographic processes which was more effective than washing in a tray. The formic acid was then washed out with water, at first chromatographically for 48 hours, and then in a tray to a neutral reaction to universal indicator. The alkaline-earth and heavy-metal ions were removed as before by successive treatment for 30 minutes of the paper with 0.5% versene solutions, pH 8.5, and then copious washings with twice-distilled water. We also washed the paper with both solvents used for separation of the nucleotides; the paper was treated with each of them successively for 48 hours by the chromatographic method. After this series of washings the two-phase chromatograms for separation of the nucleotides showed no ultraviolet-absorbing front. However, such treatment required large amounts of solvents. Therefore, we often washed the paper with formic acid, the more so because we judged from the control experiments that the eluates from equal weights of different portions of the paper gave the same very slight absorption (on extraction of 50 mg of paper with 3 ml of 0.01 N HCl the extinction at 260-240 mμ varied from 0.020 to 0.030).

Applying the Spots

For applying the spots, both in the qualitative and the quantitative experiments we used an ordinary micropipet graduated in 0.001 ml. The important factors were the specific size and the drying of the spots; when too much substance accumulated on too small an area and when the paper was dried at the start, a spot which absorbed ultraviolet remained. Therefore, 0.15 ml of trichloroacetic acid (TCA) filtrate was usually applied in a circle with a diameter of about 10 mm and each following portion was applied when visible moisture had disappeared. To obtain the fullest possible washing away of the substance from the start, before the final drying we added, as before [10], a small amount of versene solution.

Solvents

For separating the nucleotides on a one-phase chromatogram we used the alkaline solvent of Hanes and Ischerwood [12] which consisted of n-propanol – 25% NH_4OH – H_2O (60 : 30 : 10). However, as was shown earlier [10], this solvent does not separate guanosine di- and guanosine triphosphates, and only by repeated and prolonged chromatography is IMP separated from ATP. The separation of these compounds in the study of the products from anaerobic splitting and aerobic resynthesis is especially important for us. Therefore, in this work we used a two-phase chromatogram: in the first direction we passed the solvent recommended by Spirin and Bélozerskii [13] which consisted of ethanol-butanol-1 M ammonium acetate buffer, pH 3.7 (40 : 10 : 20) and for the second separation we used the alkaline solvent of Hanes and Ischerwood mentioned above. Both solvents were used in the descending direction. The first, more mobile, was passed perpendicular to the grain of the paper. The duration of separation on the Leningrad slow paper was: first direction 20 hours, second, 36 or more hours; on the Whatman No. 1, first direction 12 hours, second, 24 hours. The duration of passing the solvent was determined on the basis of the mobility of the fastest spot, which in our case was inosine. When the sheet was long enough, and allowance was made for washing the spot onto the paper, the period of separation could be increased, especially in the second direction.

Development of the Spot and Contact Printing

The development of the spot was carried out as before with the aid of the Brumberg ultrachemiscope [14] and also according to the phosphorus. The chromatograms were photographed by the contact process of Markham and Smith [15]. Before we used contact printing, it was used by Sytinskii [16] and Evreinova [17].*

As a source of ultraviolet light we used the bacteriocidal BUF 30-P lamp enclosed in a case with a special shutter. No filter improved the results and we used none of them. The distance from the light source to the panel with the chromatogram and the photopaper (Unibrom, high-contrast No. 5) was 2.3 m; the exposure was 4.5-5 seconds.

The contrast of the print can be strengthened considerably by overdeveloping the plate and later printing on Unibrom paper No. 3 or 4 (the reproductions of the chromatograms in this work were actually obtained in this way). We also obtained contact prints of the chromatograms developed for phosphorus; an ordinary incandescent lamp was the light source in this case.

*We extend thanks to T.N. Evreinova for advice on this method.

Fig. 1. The separation of nucleotides of pigeon erythrocytes by two-phase chromatography of the initial samples: 1) ATP; 2) ADP; 4) GTP; 5) GDP.

Fig. 2. The separation of nucleotides of pigeon erythrocytes by two-phase chromatography of the anaerobic samples: 1) ATP; 2) ADP; 3) AMP; 4) GTP; 5) GDP; 6) GMP; 7) IMP; 8) inosine + hypoxanthine.

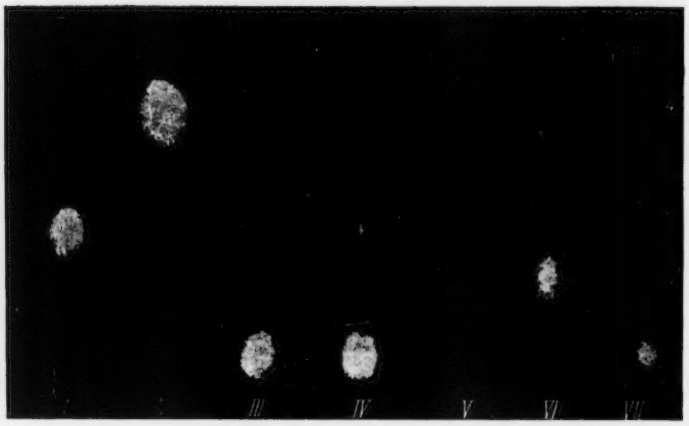

Fig. 3. Identification of inosine and hypoxanthine on a rechromatogram: 1) inosine; 2) adenosine; 3) hypoxanthine; 4) adenine. Spots: I) hypoxanthine; II) adenine; IV) adenosine; VII) inosine; V) control sample; III) anaerobic sample; IV) resynthesis sample.

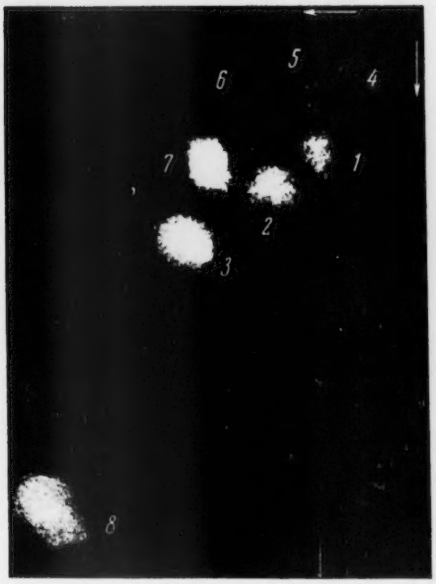

Fig. 4. The separation of nucleotides of pigeon erythrocytes by two-phase chromatography after treatment with HCN: 1) ATP; 2) ADP; 3) AMP; 4) GTP; 5) GDP; 6) GMP; 7) IMP; 8) inosine + hypoxanthine.

Fig. 5. The separation of nucleotides of pigeon erythrocytes by two-phase chromatography after treatment with DNF: 1) ATP; 2) ADP; 3) AMP; 4) GTP; 5) GDP; 6) GMP; 7) IMP; 8) inosine + hypoxanthine.

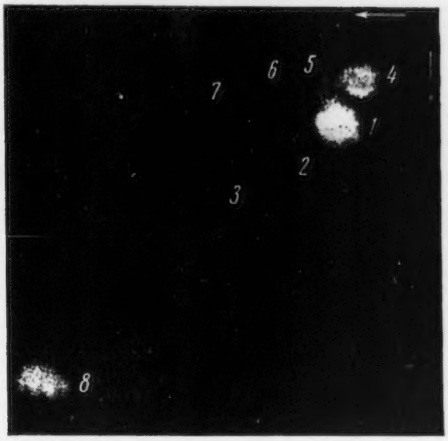

Fig. 6. The separation of nucleotides of pigeon erythrocytes by two-phase chromatography of the resynthesis samples: 1) ATP; 2) ADP; 3) AMP; 4) GTP; 5) GDP; 6) GMP; 7) IMP; 8) inosine + hypoxanthine.

Rechromatography

In the identification of the nucleotides we used rechromatography. This method is particularly useful when the solutions of the substances under investigation contain a high concentration of salts, which is the case, for example, in the nucleotide hydrolysate which contains the free bases. The presence of salts deforms the spots on the chromatogram and changes their rate of migration so that the identification of unknown substances by indicators on the chromatogram becomes impossible.

We usually carried out rechromatography as follows.

The solution which contained the substances for separation was placed on the paper and passed on in the usual way. If there was a high salt concentration in the solution, then in the ultrachemiscope washed-out spots and long fluorescent stains appeared. The spots which absorb the ultraviolet were cut out in little wedges, with an attempt to obtain those farthest from the part of the paper saturated with the salts. The separate wedges were fastened to clean strips of paper by thread; the long end of the wedge was placed in a small chromatographic vessel held on a support, and the other, pointed end was placed in contact with the clean paper at the level of the starting line of the second chromatogram. Water or a very dilute solution of acid or versene was put into the vessel (the choice of the eluent was determined by solubility relations of the substance which was transferred). When the solution had impregnated the entire wedge of paper and a spot of moisture appeared on the rechromatogram a current of air was introduced from below. The apparatus as a whole was similar in construction to that described by Wilson and Calvin [18]. Because of drying, the entrance of liquid and the substance transferred from the wedge onto the rechromatogram was speeded up. The area of the spot depended on the length of the wedge and the area of its pointed end in contact with the paper as well as the intensity and temperature of the current of air. Whatever the relation of these factors might be, sooner or later an equilibrium would be established between the inflow and evaporation of the solution and the spot became stationary in size. This permitted regulation of its size within definite limits. The transfer was continued for 15 to 60 minutes, depending on the size of the spot; the completeness of the transfer was then tested by examining the dried wedge under the ultrachemiscope. Since in the rechromatography the spot contains no salts, its progress is without interference and unknown substances can be identified with the aid of standard indicators.

Rechromatography was used under the following conditions.

1. For identification of the nitrogenous bases which occurred in unknown compounds. The eluate of the corresponding spot was dried lyophilically. The residue was hydrolyzed in 70% $HClO_4$ at 100° for one hour. Then the hydrolyzate was neutralized to a weak acid reaction and deposited on the chromatogram with simultaneous neutralization by ammonia. Separation was carried out with the solvent isopropanol-5 N HCl-ethanol (6:2:2) for 18-20 hours. The poorly separate but sufficiently clearly visible spots were cut out and rechromatographed by the process we have described.

2. For identification of the fractions obtained on an ion-exchange column.

3. For comparing and identifying spots obtained in different solvents or by the parallel use of electrophoresis and paper chromatography. In this case the corresponding spots were transferred to the same chromatogram (if necessary, along with the indicator) and then treated in the usual way.

4. If the spots on the chromatogram or the electrophoretogram were formed by two or several substances with the same mobilities in a given solvent. Under these circumstances the spots were transferred to the rechromatogram and separated by passing a solvent in which the substances had different R_f.

Conduct of the Experiments

The densely packed centrifuged erythrocytes obtained from defibrinated pigeon blood, washed with physiological salt solution, were diluted with Ringer-Krebs solution (without Ca and phosphate but with addition of 2% glucose) in the ratio 1:1. The erythrocyte suspension was placed in a special flask [3] which was supported in a rocker of a water thermostat at 37°. Anaerobic conditions were attained by repeated evacuations of the vessel and filling it with N_2, free from traces of O_2, and with 5% CO_2. For this purpose, the nitrogen from a cylinder was passed either through heated copper turnings reduced by hydrogen [3], or through a high glass column filled with an alkaline solution of hydrosulfite. The first process was more effective. The erythrocytes were usually kept under anaerobic conditions for about 30 minutes. At the expiration of this time, samples were

collected for preparing TCA extracts (anaerobic samples). Then the erythrocyte suspension was saturated with oxygen in the cold and incubated 60 minutes more at 37°. We then again collected samples (resynthesis samples). For obtaining the extracts, the initial samples of erythrocyte suspension were kept in the thermostat with passage of air like the anaerobic samples. The TCA extracts in all cases were obtained by 10-minute extraction of the erythrocyte suspension [11] with an equal volume of 1.5% TCA in the cold. At the start of the chromatogram we added 0.15 ml of TCA extract. Washing the precipitate, after precipitation of the protein, was carried out only to control complete extraction, and for all chemical and chromatographic determinations the TCA extract was used directly.

Other Determinations

The nucleotides were determined quantitatively by the spectrophotometer; phosphorus was determined according to Fiske-Subbarow and also by the modified method of Lowry [19], pentoses according to Maybaum in the Lyubimova modification [20], nitrogen by digesting according to Kjehldahl with later distillation. For the determination of nucleotides and also phosphorus compounds which did not contain purine bases, by their phosphorus, we used the reaction of Hanes and Ischerwood in the modification of Burrows and co-workers [21]. All the values were calculated to 1 ml of densely packed centrifuged cells.

EXPERIMENTAL

Qualitative Evaluation of the Chromatogram

The qualitative results of our experiments were studied by contact photographic chromatography of initial, anaerobic, and resynthesis samples.

On the chromatogram of the initial samples (Fig. 1) the largest and clearest spot belongs to ATP (1), the second in size comes from GTP (4). We can also see moderately intense spots for ADP (2) and sometimes AMP. The latter compound and also adenosine pentaphosphate are clearly seen on the one-phase chromatogram, but on the two-phase chromatogram all the spots occupy a greater area and their absorption is lessened so that compounds present in small concentrations cease to be perceptible to the eye when examined in the chemiscope and do not reproduce on the contact print. Guanyl derivatives are also represented by weak spots of GDP (5).

The chromatogram of the anaerobic samples (Fig. 2) is characterized by a deep change in the nucleotide composition of the erythrocytes resulting from the anaerobic conditions. This is shown in the change in intensity of the spots which were seen earlier and in the appearance of new ones. The transformation of the adenylic nucleotides under anaerobic conditions includes a marked decrease in intensity of the ATP spot (1), and increase in the ADP spot (2), and a strengthening (where it had appeared in the initial sample) or appearance of a spot for AMP (3). The changes in the guanylic system have an analogous, though less strongly expressed character, since the spot for GTP is decreased (4), the density of the spot corresponding to GDP (5) is increased, and a clear spot for GMP (6) appears. We should state that the violet fluorescence of the guanylic derivatives which occurs when the chromatogram is treated with hydrogen chloride is an especially sensitive test (for this it is only necessary to keep the chromatographic paper over hydrochloric acid). The spots of the guanosinepolyphosphates are clearly visible to the eye when neither the contact prints nor the photometric study of the eluate give results. The spot of guanylic acid in the anaerobic sample (4) is always present, but often there can also be seen a violet fluorescing spot with a high R_f which, to judge by its position, corresponds to guanosine (this is never obtained on the print). Two more spots also appear on the chromatogram. One of these is inosinic acid (7), whose identification is not difficult, since the chromatographic behavior of this compound was studied before [10]. The second spot was studied more carefully, since it has never been described. The high value for R_f of this spot and also its appearance only on the chromatogram from the anaerobic sample, and the dependence of its intensity on the duration of the anaerobic conditions show clearly that it comes from some product of splitting ATP. Analysis showed that phosphorus is not present in the spot, but ribose is, and the single base which it contains is a purine with an absorption maximum corresponding to hypoxanthine. All this led to the conclusion that the second spot comes from inosine formed in the pigeon erythrocytes under anaerobic conditions. However, the analytical data still do not exclude the presence of hypoxanthine. Final identification of the spot was made by its rechromatography with a solvent of n-butanol, saturated with water – 25% NH_4OH in the ratio 100 : 1 [22]. As an indicator, in addition to hypoxanthine and some other nitrogen bases we used inosine, obtained by the action of a specific deaminase on adenosine.* The rechromatogram is shown in Fig. 3. These spots and also those in the resynthesis

*The enzyme preparation was kindly presented to us by G.A. Kritskii.

TABLE 1

Nucleotides, P_i, P_{total}, and Total Nitrogen of TCA Extracts of Pigeon Erythrocytes (in μmoles per ml erythrocytes)

Expt. No.	Sample	P_i	P_{total}	Total nitrogen	Sum of nucleotides	Adenylic system				Inosinic system			Guanylic system			
						ATP	ADP	AMP	sum	IMP	inosine + hypoxanthine	sum	GTP	GDP	GMP	sum
1	Initial	1,2	41,58	117,50	5,0	0 4,23	0,29	0	4,49	0	0	0	0,39	0,12	0	0,51
	Anaerobic	3,28	50,49	150,55	6,54	3,2	1,21	0,55	4,99	0,08	0,87	0,95	0,32	0,21	0,07	0,60
	Resynthesis	3,07	45,93	146,88	4,90	3,58	0,18	0,05	3,81	0	0,52	0,52	0,53	0,04	0	0,57
2	Initial	1,62	31,48	105,10	5,39	4,08	0,46	0,12	4,66	0	0	0	0,65	0,08	0	0,73
	Anaerobic	5,07	54,25	144,51	7,45	3,35	1,16	0,48	4,99	0,16	1,89	2,05	0,37	0,04	0	0,41
	Resynthesis	4,07	54,87	124,80	6,46	4,25	0,27	0,25	4,77	0,04	1,14	1,18	0,50	0,01	0	0,51
3	Initial	2,2	49,10	98,42	4,76	4,02	0,22	0	4,24	0	0	0	0,46	0,06	0	0,52
	Anaerobic	10,0	54,64	118,03	6,94	1,57	0,97	0,76	3,30	1,12	1,99	3,11	0,31	0,12	0,10	0,53
	Resynthesis	8,56	48,70	109,52	5,56	2,55	0,09	0	2,64	0,09	2,09	2,18	0,66	0,08	0	0,74
4	Initial	1,70	48,90	115,24	5,16	4,37	0,23	0	4,61	0	0	0	0,44	0,12	0	0,56
	Anaerobic	4,34	54,25	126,24	6,83	3,34	1,08	0,46	4,88	0,16	1,28	1,44	0,41	0,10	0	0,51
	Resynthesis	3,80	51,48	104,59	5,49	3,95	0,27	0,17	4,39	0	0,53	0,53	0,50	0,07	0	0,57

samples contain inosine and traces of free hypoxanthine. Thus, we consider it to be established that under anaerobic conditions in pigeon erythrocytes, three derivatives of the inosine series are formed to a considerable extent: IMP, inosine and hypoxanthine.

The nature of the splitting of ATP does not depend on the process by which anaerobic conditions are attained, as Fig. 4 shows, where we give the chromatogram of the nucleotides of pigeon erythrocytes incubated with HCN. We also obtained an analogous picture when we poisoned the erythrocytes with a typical dissociating poison, dinitrophenol (Fig. 5). The similarity of these chromatograms is so evident that there is no need for any explanation.

We now turn to the chromatogram of the resynthesis sample (Fig. 6), that is, the sample of erythrocytes incubated in an atmosphere of O_2 after a preliminary period of anaerobic conditions. The chief result of the restoration of respiration to the erythrocytes is the resynthesis of ATP which is shown on the chromatogram by the increase in the ATP spot. The ADP spot, on the contrary, is strongly decreased; the spots for AMP and IMP either decrease strongly or disappear altogether. The spot for inosine becomes less but still has a fairly great size. The same changes occur in the guanylic system as in the adenylic, namely: increase in triphosphate content and decrease in diphosphate. Thus the resynthesis chromatograms return to a considerable extent to the original picture due to the processes of aerobic restoration, though the spots for the inosine derivatives remain as a reminder of the sharp changes in erythrocyte metabolism during the period of anaerobic conditions.

Quantitative Determinations of the Nucleotides Separated by Chromatography

The data for the quantitative determination of nucleotides of nucleated erythrocytes in relation to respiration are given in Table 1.

The total nucleotide content in different experiments varied from 4.76 to 5.39 μmoles, which agreed approximately with the earlier results [10] averaging 5.11 μmoles, although we did not study adenosine pentaphosphate, whose concentration was small, averaging about 0.22 μmoles. In these experiments, compared to the previous determinations [10], there was somewhat more ATP and, correspondingly, less ADP and AMP.

When considering the change in content of nucleotides depending on respiration we should observe that we did not tend to obtain extreme anaerobic conditions or to maintain them for a long time, since

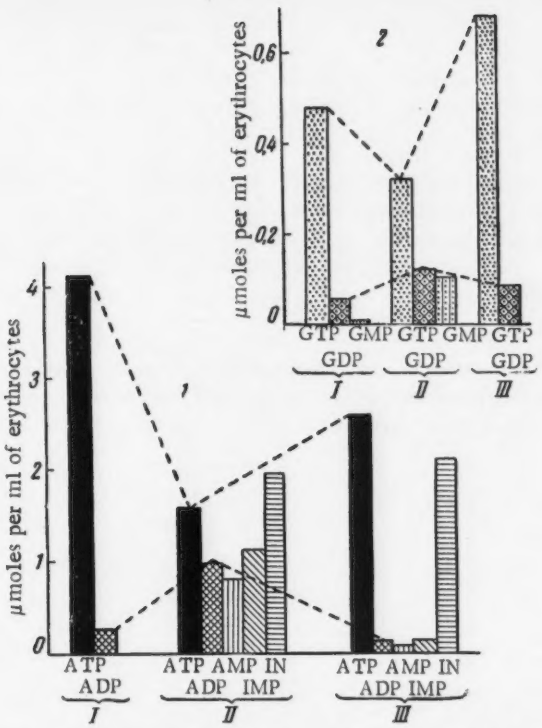

Fig. 7. Behavior of adenylic (1) and guanylic (2) nucleotides of pigeon erythrocytes in anaerobic splitting and aerobic resynthesis: I) initial sample; II) anaerobic sample; III) resynthesis sample.

TABLE 2

Successive Changes in Nucleotide Content in Pigeon Erythrocytes in Relation to Respiration (in μmoles per ml of erythrocytes)

Expt. No.	Sample	Adenylic system			Inosinic system	Sum of inosinic and adenylic systems
		ATP	ADP + + AMP	sum		
1	Anaerobic	−0.97	+1.47	+0.5	+0.95	+1.45
	Resynthesis	+0.35	−1.53	−1.18	−0.043	−1.61
2	Anaerobic	−0.73	+1.06	+0.33	+2.05	+2.38
	Resynthesis	+0.90	−1.12	−0.22	−0.87	−1.09
3	Anaerobic	−2.45	+1.51	−0.94	+3.11	+2.17
	Resynthesis	+0.98	−1.64	−0.66	−0.93	−1.59
4	Anaerobic	−1.03	+1.31	+0.28	+1.44	+1.72
	Resynthesis	+0.61	−1.10	−0.49	−0.91	−1.40

then the splitting of ATP would have gone too far and worsened the conditions of resynthesis which we had considered still earlier [3]. On the other hand, in erythrocytes, whose hemoglobin contains a considerable amount of loosely bound oxygen, it is hard to obtain the required anaerobic conditions. Therefore, in spite of the outwardly comparable standard conditions, it was practically impossible to attain the same degree of anaerobic

state, which explains some of the variations in quantitative results, depending on how deep the anaerobic conditions were. Since the main problem of our investigation was to solve the question of whether under these experimental conditions there is true oxidative phosphorylation in pigeon erythrocytes, we treated the results in Table 1 chiefly from this point of view. We can see (Table 1) that the content of P_i rises from the initial 1.2-2.2 μmoles depending on the anaerobic conditions to 3.28-10 μmoles and then falls with resynthesis to 3.07-8.56 μmoles. Hence, decrease in P_i content occurs during resynthesis. The nucleotide content changes in accord with this picture as is seen visually on the chromatogram. In Fig. 7 we express graphically the results of experiment 3 (Table 1).

The data of Table 1 show that the guanylic system does not give marked or simple changes. There is not always an increased content of GMP under anaerobic conditions, the GDP content sometimes rises, sometimes falls; however, the content of GTP always falls in anaerobic conditions and increases in resynthesis. It is possible that in the quantitative determination of the guanylic derivatives we are dealing with concentrations which lie close to the boundary of sensitivity of the method, which would explain some of the scattering in the values of GDP and GMP.

In Table 2 we give the successive quantitative changes of the adenylic and inosinic systems, that is, the increase or decrease in content of the corresponding components when we compare the anaerobic with the initial samples and then the resynthesis with the anaerobic.

Depending on the severity of the anaerobic conditions the ATP content decreases by 2.45-0.73 μmoles, and in resynthesis increases by 0.35-0.98 μmoles. The sum of ADP and AMP in the first case, on the contrary, increases by 1.06-1.57 μmoles and in the second, falls by 1.10-1.64 μmoles.

The content of inosinic derivatives under anaerobic conditions increases by 0.95-3.11 μmoles and in an atmosphere of oxygen decreases by 0.43-0.93 μmoles; this decrease occurs chiefly because inosinic acid disappears almost entirely (Table 1) and to a much smaller degree because of inosine. We could not detect an accumulation of IDT and ITP in the anaerobic and resynthesis samples.

We should observe that Fleckenstein and his group [23-26], in study of the problem of active transport of cations, investigated nucleotides and phosphate esters of pigeon erythrocytes and also the effect of HCN and DNP on their metabolism. The data of Fleckenstein with respect to nucleotide content and general character of its transformations under the influence of HCN and DNP agreed with ours. However, these authors did not observe that when phosphorylation stopped, inosine and hypoxanthine were formed. Perhaps this was because they identified substances on the chromatogram chiefly by their phosphorus, and the movement of the inosine and hypoxanthine could go unnoticed from off the chromatogram after prolonged passage of solvent.

Nucleotide Balance

When we began the quantitative investigation of the change in nucleotides in the erythrocytes with respect to respiration, we expected to obtain an exact balance of all the transformations, since we believed that the total nucleotide content in the TCA extracts under different conditions should not change much. However, we could not get such agreement, although Fleckenstein and his group succeeded in establishing an exact balance in experiments on human and pigeon erythrocytes [26] and on brain tissue [27]. Actually, the sum of the nucleotides of the TCA extract of erythrocytes (Table 1) increased under anaerobic conditions by 1.54-2.18 μmoles and fell in resynthesis by 0.98-1.64 μmoles, which is beyond the possible experimental error. The validity of this effect is confirmed by the parallel increase in total P and total N, although the increase of these, and especially the N, exceeds the ratio of 1:3:5 expected for a nucleoside triphosphate. Thus the reality of this fact cannot be doubted. Analysis of the data of Tables 1 and 2 shows that the participation of the guanylic system in the formation and removal of excess nucleotides is slight, and that this process is connected almost entirely with the adenylic and inosinic fractions. In fact, their total content (Tables 1 and 2) increases under anaerobic conditions by 1.45-2.38 μmoles and decreases in resynthesis by 1.09-1.61 μmoles, which fully covers the change in total nucleotide balance. When we consider the contribution of the adenylic system to the total nucleotide balance, we see that its changes make up only a small part of the total nucleotide changes. This can be understood by considering the contradiction in signs of change in ATP content on the one hand, and of ADP + AMP on the other. Also, under anaerobic conditions there occurs a "leakage" of adenylic nucleotides into the inosine system and the observed increase in content of the latter is correctly considered in connection with the change in the former. In aerobic resynthesis, both systems apparently function independently of each other. The following important features are evidently characteristic of changes in the adenylic system: in anaerobic splitting the

decrease in ATP is greater than the increase in the ADP + AMP fraction (intense "leakage" into the inosine system occurs); under mild anaerobic conditions (experiments 1, 2 and 4) the increase in ADP + AMP content surpasses the loss in ATP. All this is easy to follow from the data of Table 1 and especially from Table 2. The change in content of adenylic nucleotides with respiration cannot be explained only by regroupings in the limits of the nucleotides which we have studied, but depends, in our opinion, on exchange of the latter with the acid-soluble nucleotide fraction which is precipitated by TCA.

DISCUSSION OF RESULTS

When we consider these results from the point of view of our problem, we can see that our data undoubtedly show that in the restoration of respiration in pigeon erythrocytes after a preliminary anaerobic period true oxidative phosphorylation occurs (binding of inorganic phosphate, phosphorylation of ADP and formation of ATP). The data once more confirm that in 1930-1932 Engel'gardt actually discovered oxidative phosphorylation in cells with a predominantly aerobic metabolism [1-3]. The nature of the products of aerobic resynthesis of nucleated erythrocytes can be considered to be finally established: fundamentally it is ATP; we found no sign of formation of IDP, ITP, or other nucleotides. In part, however (in amounts not surpassing 10%) we found an increase in easily hydrolyzed P which showed the resynthesis of GTP. However, the guanylic derivatives seem to be a system of secondary importance both in quantitative relations and in the order in which their changes occur. They react under anaerobic conditions only after splitting has already gone rather far in the adenylic system. This agrees with the data obtained by Fleckenstein et al. [26] in a study of the renewal of the phosphate groups of ATP and GTP with the aid of P^{32}. They showed that if the renewal of P^{γ} of ATP is taken as 1000, then the renewal of P^{β} in an equal time interval is about 800 and the renewal of P^{γ} and P^{β} of GTP is only 500. Whether the transformations of GTP are independent of the adenylic system or its phosphorus is exchanged with ADP with the aid of nucleoside diphosphokinase [28, 29] is unknown.

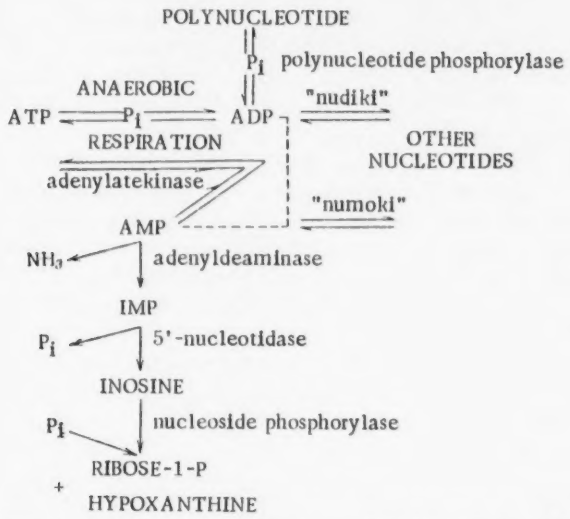

Fig. 8. Scheme of transformation of nucleotides in erythrocytes.

The assumed mechanism for the transformations of nucleotides of nucleated erythrocytes in relation to respiration is shown in Fig. 8. Under anaerobic conditions in the erythrocytes there is accumulation of ADP since, on the one hand ATP is used in one or another endergonic process, and on the other, cessation of respiration blocks the process of oxidative phosphorylation of ADP. Anaerobic glycolysis of the erythrocytes is evidently little effective in this respect and cannot replace the energy of respiratory phosphorylation of the erythrocytes which, to judge by the action of DNP, occurs preferably in the respiratory chain. ADP under the influence of adenylate kinase, which, according to the data of Fleckenstein et al. [26], is distinguished by its high activity in pigeon erythrocytes, is transformed into ATP and AMP. The latter is deaminated by the highly active adenyl

deaminase [7, 8] and forms inosinic acid, the accumulation of which is prevented by 5-nucleotidase which transforms it into inosine. Metabolism of the latter evidently proceeds slowly and as a result of phosphorylation catalyzed by nucleoside phosphorylase gives ribose-1-phosphate and hypoxanthine. Further transformations of the inosine compounds were not followed, and we do not know how much and how the ribose-1-phosphate which is formed is used. Numerous studies of the metabolism of ribose-1-phosphate and the nucleoside diphosphorylase in erythrocytes in connection with the preservation of blood [30-36] allow us to assume that the phosphate ester can be used in the erythrocytes in anaerobic metabolism as a source of energy.

In aerobic resynthesis there is phosphorylation of ADP connected with respiration, and not the myokinase reaction $2ADP \rightleftharpoons ATP + AMP$. The observed binding of P_i and the almost complete disappearance of AMP in anaerobic resynthesis are the chief pieces of evidence for this. In the myokinase reaction we would find the contrary effect. The transformation of AMP into IMP under these conditions could not mask the myokinase reaction since it has been shown repeatedly and without doubt that under aerobic conditions in pigeon erythrocytes the accumulation of NH_3 does not occur [5, 7]. This explains the aerobic blocking of the deamination of AMP which is normal for anaerobic conditions. The myokinase reaction nonetheless does play an auxiliary part in aerobic resynthesis, not in the removal of ADP, but on the contrary, in its formation. Evidently the following two reactions occur, of which the second is the oxidative phosphorylation:

$$AMP + ATP = 2ADP$$
$$2ADP + 2P = 2ATP$$

$$AMP + 2P = ATP$$

This is the most probable path of removal of AMP; ATP here plays the part of a coenzyme. Whether inosinic acid is transformed into adenylic derivatives through adenyl succinate [36-38] is difficult to say. The scheme also shows the possibility of exchange of phosphate between ADP and AMP and other nucleotides, such as guanylic compounds with the help of "nudiki" and "numoki."*

Reversible exchange between low-molecular-weight fractions of the TCA extract and high-molecular-weight polynucleotides precipitated by TCA from pigeon erythrocytes in relation to respiration is very probable in view of the results of a number of investigations. Here we find the elegant work of Ochoa, Grunberg-Manago et al. [39, 40] and the sufficiently well-known work of Kornberg et al. [41]. Investigations [42-45] carried out with various animal preparations or tissues have also indicated the possibility of including nucleotides in polynucleotides without preliminary breakdown into simpler fragments. Some literature directly indicates the presence of a connection between ATP synthesis, high-molecular-weight polynucleotides, and oxidative phosphorylation; this has been shown, for example, by Pinchot [46] and Allfrey and Mirsky [47]. Depolymerization of high-molecular-weight polynucleotides, to judge by such data, proceeds by phosphorolysis or pyrophosphorolysis, and is accompanied by binding P_i. If depolymerization of the TCA precipitated polynucleotides actually occurs in the erythrocytes with formation of free nucleotides, and this process is also phosphorylic, then in the anaerobic samples we should have a difference between the analytically established increase in P_i and the calculated increase on the basis of which we could find the increase or decrease in nucleotide content. If phosphorolysis occurs there should be a deficit of phosphorus corresponding to the total increase of nucleotides. Calculation actually shows that the deficit occurs and is established in experiments 1, 2, 4 (Table 1) at 3.08, 3.96, 3.16 μmoles P. If we assume that under anaerobic conditions phosphorolysis of polynucleotides occurs, there would be a binding of phosphorus corresponding to 2.94, 4.12, 3.34 μmoles P which agrees well with the first figures.

As an example we give the calculation of the balance for one of the experiments.

Amount of nucleotides in the initial sample 5.00 μmoles

Amount of nucleotides in the anaerobic sample 6.54 μmoles

Increase 1.54 μmoles

The phosphorolytic formation of these from polynucleotides would require $1.54 \times 2 = 3.08$ μmoles P (calculated as triphosphate).

Now we calculate the amount of P which should be formed, starting from conversion of the nucleotides in the separate systems (considering that all the transformations begin with triphosphate).

*The terms "nudiki" and "numoki" are peculiar to Russian nomenclature. They probably indicate nucleotide diphosphokinase and nucleotide monophosphokinase respectively. — Publisher.

Adenylic system:	0.92 + (0.55 × 2) = 2.02 μmoles
Inosinic system:	(0.08 × 2) + (0.87 × 3) = 2.77 μmoles
Guanylic system:	0.09 + (0.07 × 2) = 0.23 μmoles
	Total 5.02 μmoles

The actual increase in total P_i in this experiment was 2.08 μmoles; thus the deficit is 5.02 − 2.08 = 2.94 μmoles, expended in phosphorolysis. This is very close to 3.08 μmoles.

The idea of phosphorylation of the polynucleotides is also supported by the fact that in the erythrocytes only adenylic nucleotides are formed. Such a possibility is shown by the work of Grunberg-Manago et al. [40, 41] and Pinchot [46].

On the basis of the above, phosphorylative liberation of adenylic nucleotides from some sort of polynucleotide can be considered entirely probable (polynucleotides of pigeon erythrocytes under anaerobic conditions, and, on the contrary, their inclusion in polynucleotides in aerobic resynthesis).

SUMMARY

In experiments on pigeon erythrocytes we have shown that under anaerobic conditions and also with separation of respiration and phosphorylation there is splitting of ATP and GTP, accumulation of the corresponding di- and monophosphates and products of their further splitting.

We have repeated and studied qualitatively and quantitatively by paper chromatography the relation of respiration and phosphorylation which was first observed by Engel'gardt in the restoration of respiration of erythrocytes after preliminary anaerobic treatment. It was shown that the products of aerobic resynthesis in nucleated erythrocytes are ATP and GTP, formed by oxidative phosphorylation. Among the products of splitting we have noted considerable amounts of inosine.

We have established an increase in total content of adenylic and inosinic nucleotides under anaerobic conditions and their decrease in aerobic resynthesis. The total nitrogen and total phosphorus of the TCA extracts are changed in an analogous manner. We have suggested that the excess nucleotides are formed under anaerobic conditions because of depolymerization of the polynucleotides which are precipitated by TCA and that in aerobic resynthesis the reverse process of including free nucleotides in the polynucleotides occurs.

LITERATURE CITED

[1] V.A. Engel'gardt, Biochem. Z. 227, 16 (1930).

[2] V.A. Engel'gardt, Kazansk. Med. Zh. 27, 496 (1931).

[3] V.A. Engel'gardt, Biochem. Z. 251, 343 (1932).

[4] A.A. Baev, Doklady Akad. Nauk SSSR 4, 69 (1934).

[5] V.A. Engel'gardt and A.A. Baev, Biokhimiya 1, 8 (1936).

[6] V.A. Engel'gardt, Izv. AN SSSR, No. 4, 647 (1936).

[7] A.A. Baev, Biokhimiya 2, 454 (1937).

[8] A.A. Baev, Transformations of Adenosine Triphosphoric Acid in Nucleated Erythrocytes of Birds in Connection With Respiration. Dissertation [in Russian] (Moscow, 1946).

[9] V.A. Belitser, Chemical Transformations in Muscle [in Russian] (Medgiz, Moscow-Leningrad, 1940).

[10] T.V. Venkstern and A.A. Baev, Biokhimiya 22, 1043 (1957).*

[11] L.V. Eggleston and R. Hems, Biochem. J. 52, 156 (1952).

[12] C.S. Hanes and F.A. Ischerwood, Nature 164, 1107 (1949).

[13] A.S. Spirin and A.N. Belozerskii, Doklady Akad. Nauk SSSR 113, 650 (1957).*

[14] E.M. Brumberg and E.A. Materova, Collection: Chromatography [in Russian] (Izd. Leningradsk. Un-ta, Leningrad, 1956).

*Original Russian pagination. See C.B. translation.

[15] R. Markham and J.D. Smith, Biochem. J. 45, 294 (1949).

[16] I.A. Sytinskii, Biokhimiya 21, 359 (1956).*

[17] T.N. Evreinova, L.P. Ermolaeva and A.M. Gerasimova, Doklady Akad. Nauk SSSR 118, 334 (1958).**

[18] A.J. Wilson and M. Calvin, J. Amer. Chem. Soc. 77, 5948 (1955).

[19] O.H. Lowry et al., J. Biol. Chem. 207, 1 (1954).

[20] M.N. Lyubimova, Enzymatic Properties of the Myosin of Striated Muscle, Dissertation [in Russian] (Moscow, 1957).

[21] S. Burrows, S.M. Grylls and J.S. Harrison, Nature 170, 800 (1952).

[22] W.S. MacNutt, Biochem. J. 50, 384 (1952).

[23] A. Fleckenstein and E. Gerlach, Arch. expl. Pathol. und Pharmakol. 219, 531 (1953).

[24] E. Gerlach, Arch. expl. Pathol. und Pharmakol. 222, 128 (1956).

[25] A. Fleckenstein, E. Gerlach and J. Janke, Schweiz. med. Vochenschr. 86, 1041 (1956).

[26] E. Gerlach, A. Fleckenstein and K.J. Freundt, Naturwissenschaften 43, 522 (1956); Pflügers Arch. 263, 682 (1957).

[27] E. Gerlach, H.I. Döring and A. Fleckenstein, Pflügers Arch. ges. Physiol. 266, 266 (1958).

[28] P. Berg and W. Joklik, Nature 172, 1008 (1953); J. Biol. Chem. 210, 657 (1954).

[29] H.A. Krebs and R. Hems, Biochim. biophys. acta 12, 172 (1953); Biochem. J. 61, 435 (1955).

[30] B.W. Gabrio and F.M. Huennekens, Biochim. biophys. acta 18, 585 (1955).

[31] F. Lionetti, Biochim. biophys. acta 18, 443 (1955).

[32] D. Rubinstein, S. Hashket and O.F. Denstedt, Canad. J. Biochem. and Physiol. 34, 61 (1956).

[33] F. Lionetti et al., J. Biol. Chem. 220, 467 (1956).

[34] F. Lionetti et al., J. Biol. Chem. 229, 817 (1957).

[35] A. Hock and G. Huber, Z. physiol. Chem. 308, 116 (1957).

[36] C.E. Carter and L.H. Cohen, J. Amer. Chem. Soc. 77, 499 (1955).

[37] R. Abrams and M. Bentley, J. Amer. Chem. Soc. 77, 4179 (1955).

[38] J. Lieberman, J. Amer. Chem. Soc. 78, 251 (1956).

[39] M. Grunberg-Manago, P.J. Ortiz and S. Ochoa, Biochim. et biophys. acta 20, 269 (1956).

[40] M. Grunberg-Manago, Biokhimiya 23, 307 (1958).*

[41] A. Kornberg, Advances Enzymol. 18, 191 (1957).

[42] E. Goldwasser, J. Amer. Chem. Soc. 77, 6083 (1955).

[43] C. Heidelberger et al., Biochim. et biophys. acta 20, 445 (1956).

[44] E.S. Canellakis, Biochim. et biophys. acta 25, 217 (1957).

[45] M. Edmond and R. Abrams, Biochim. et biophys. acta 26, 226 (1957).

[46] G.B. Pinchot, J. Biol. Chem. 229, 1 (1957).

[47] V.G. Allfrey and A.E. Mirsky, Proc. Nat. Acad. Sci. USA 43, 589 (1957).

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**See English translation.

THE REACTION OF ACETYLCHOLINE WITH CHOLINE RECEPTORS IN TISSUE HOMOGENATES

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The reaction of acetylcholine with effector muscle fibers is a complex process in which the first reaction occurs between acetylcholine and the choline receptors, and the final effect, change in muscle function, is evidently connected with an effect on the metabolism of the act of contraction [1]. At the present time, in studies on the frog heart, some data have been obtained on the biochemical nature of this reaction. Thus, the kinetics of the reaction of acetylcholine with the choline receptors has been submitted to the equation of Michaelis and Menton [2]; this reaction depends on the presence of free sulfhydryl groups [3, 4] and has a definite optimum temperature [5]. With the action of high temperature there occur reversible and irreversible heat inactivation of the choline receptors [2]. These results confirm the similarity between the reactions of acetylcholine and choline receptors and enzymatic processes.

The question arises how great is the similarity: whether the reaction of acetylcholine with the choline receptors proceeds in part only in the structural tissues, like an enzymatic reaction, or in nonstructural homogenates. Evidently the observation of this reaction in homogenates would ease considerably the problem of isolating and identifying the properties of specific proteins which exist in intimate relation between the mediators and the metabolic substances of the effector organs.

It was shown in previous work [4, 6] that when acetylcholine acts on the heart muscle, its characteristic action is to lower the reactivity of the tissue sulfhydryl groups. In experiments on the whole ventricle the decrease in reactivity is shown by the lessened effectiveness of the antiacetylcholine action of the thiol poison, $HgCl_2$. In homogenates prepared from the ventricle of the frog heart, the decrease in activity of the SH groups is seen by amperometric titration.

In the present work we have tried to explain how much the lowering in activity of SH groups in homogenates under the influence of acetylcholine is characteristic just for the reaction of this substance with choline receptors, and that it is not a side reaction of acetylcholine. In the experiments on the frog heart we studied the effect of acetylcholine on the reactivity of the SH groups under the influence of various factors: irreversible destruction of choline receptors (heat of denaturation [2, 5]) and reversible suppression of the sensitivity of the effector organ to acetylcholine (lowering the temperature to 0° [5], action of atropine).

METHODS

Ventricles of the hearts of several frogs were washed with Ringer solution and ground in a Potter homogenizer for 10 minutes with constant ice cooling. The resulting homogenate was diluted with physiological saline (0.65% NaCl) so that 1 ml of liquid contained 75 mg of crude tissue. First, for inactivation of the cholinesterase in the homogenate we added phosphacol (final concentration $1 \cdot 10^{-5}$ g/ml). We placed 1 ml of homogenate in a reaction vessel which contained 29 ml of physiological saline and 5-10 minutes before beginning the titration we added 3 mg of neutralized acetylcholine chloride. Titration of the SH groups of the homogenate was carried out by the method of mercurimetric titration [7] with some modifications which have been described before [8]. Irreversible inactivation of the choline receptors was carried out by heating the intact ventricle three times to 40°

for 3.5 minutes with intervals of 30-60 minutes [2]. The degree of inactivation of the choline receptors was controlled by the reaction on acetylcholine 18-20 hours after the last treatment. Titration of SH groups at low temperature was carried out in melting ice.

RESULTS

In Fig. 1 we give four curves typical of the mercurimetric titration of thiol groups of the homogenate of cardiac muscle; these consist of three parts. The beginning of the curve (horizontal part), when the addition of mercury ions does not change the value of the diffusion potential, corresponds to full binding of the mercury ions by the sulfhydryl groups. The second part of the curve (beginning of the rise) expresses the partial binding of Hg ions. Finally, the third part of the curve of titration (straight line with a slope to the abscissa) shows the directly proportional relation between amount of added mercury and size of the diffusion potential when all the SH groups are fully blocked. According to the results of Kolthoff [7] the point of intersection of the prolongation of the first and third parts of the curves corresponds to the amount of mercuric chloride which enters the titration.

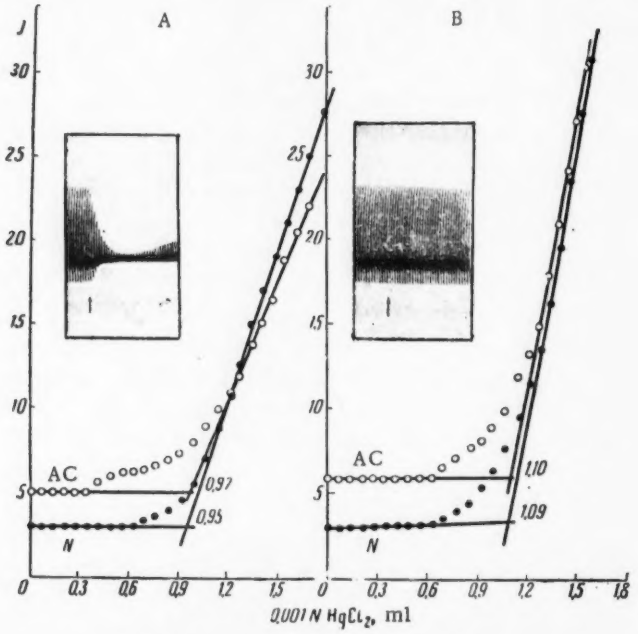


Fig. 1. Curves of mercurimetric titration of SH groups from homogenates of the ventricles of frog hearts normally (N) and under the action of acetylcholine (AC) at final concentration $1 \cdot 10^{-4}$ g/ml: A) ventricle homogenate with normal sensitivity to acetylcholine (kymograph record); B) ventricle homogenate whose sensitivity to acetylcholine has been suppressed by heat treatment (kymograph record). Ordinate – current strength in microammeter divisions. Abscissa – amount of 0.001 N solution of $HgCl_2$. Arrow – introduction by cannula of acetylcholine at a concentration of $1 \cdot 10^{-6}$ g/ml.

When the cardiac muscle homogenate is titrated in the presence of acetylcholine at a concentration of $1 \cdot 10^{-4}$ g/ml, only the shape of the titration curve is changed; the total number of SH groups remains as before. Under the influence of acetylcholine, the rise in the curve begins considerably earlier. Then the curve becomes more sloping and sometimes returns to a horizontal line above the original line (Fig. 1A). The transfer of the curve to a new level (the "acetylcholine wave") indicates that in the presence of acetylcholine a part of the tissue SH groups become less reactive and a definite amount of mercuric chloride must be in the solution for their reaction with mercury ions.

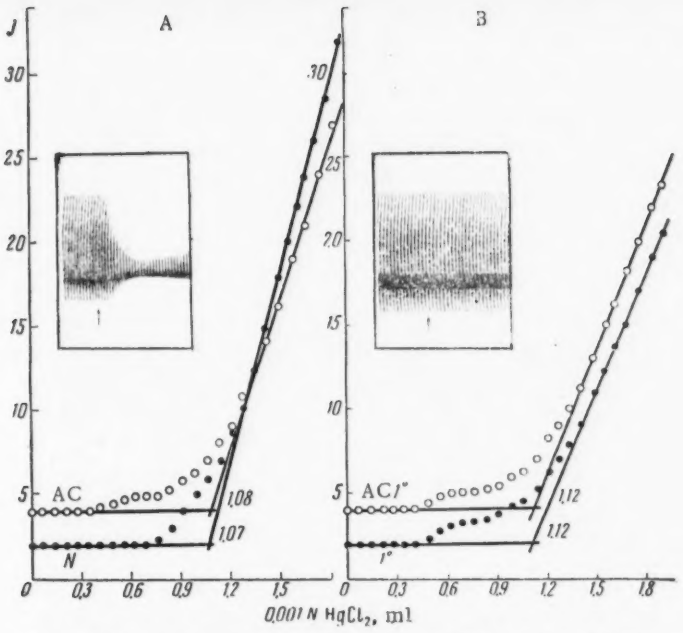


Fig. 2. Curve of mercurimetric titration of thiol groups of homogenates from frog-heart ventricles in the normal state (N) and under the action of acetylcholine (AC): A) titration of the homogenate at 18°. At this temperature the intact ventricle is sensitive to acetylcholine (kymograph record); B) titration of homogenate at 1° [the intact ventricle is not sensitive to acetylcholine (kymograph record)]. Designations the same as in Fig. 1.

It has further been shown that the acetylcholine effect in the homogenate depends on the concentration of acetylcholine. At concentrations on the order of 10^{-5} g/ml the acetylcholine wave is much weaker, and acetylcholine in concentrations on the order of 10^{-6} g/ml or below has no effect on the curve of amperometric titration. These results agree with those of previous experiments on the determination of the influence of acetylcholine on the reactivity of SH groups of choline receptors of whole frog ventricles, in which it was shown that the reactivity of thiol groups of thiol receptors is considerably decreased when acetylcholine acts in concentrations about equaling or surpassing the concentration of $HgCl_2$, and is not changed when lower concentrations are used [4]. After irreversible heat inactivation, the myocardium structures which are sensitive to acetylcholine do not show the acetylcholine wave in the curve of mercurimetric titration; acetylcholine does not change the titration curve of the homogenate SH groups when the homogenate is prepared from the ventricles with inactivation of the choline receptors (Fig. 1B). These results show that acetylcholine decreases the reactivity of SH groups just in the choline receptors, and the change is the same as in the whole cardiac muscle.

In the next series of experiments we studied the effect of low temperatures on the titration curve of homogenate SH groups in normal cases and in the presence of acetylcholine. The literature describes changes in the properties of SH groups titrated in the cold. Thus, according to the results of Muriyama [10], when the proteins of the blood of warm-blooded animals are titrated at low temperature, there is found a decrease in the total number of thiol groups. In our experiments the total number of SH groups did not change, but the reactivity of part of the homogenate SH groups was lowered. Just as when acetylcholine acted at room temperature, the rise in the titration curve of cooled homogenate began much earlier, but the general form of the cooled curve differed little from the acetylcholine curve. The addition of acetylcholine did not change the titration curve of SH groups in the cold: the rise in the curve occurs in the same way as in the case of titration of the cooled homogenate without acetylcholine (Fig. 2).

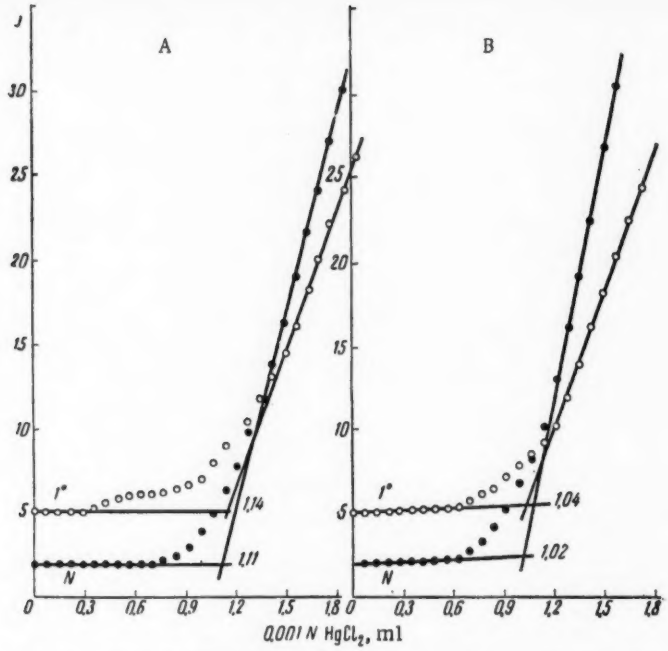


Fig. 3. Curve of mercurimetric titration of SH groups in ventricle homogenates at 18° (N) and at 1°: A) titration of ventricle homogenate with normal sensitivity to acetylcholine; B) titration of ventricle homogenates whose sensitivity has been irreversibly removed by heat treatment. Designations the same as in Fig. 1.

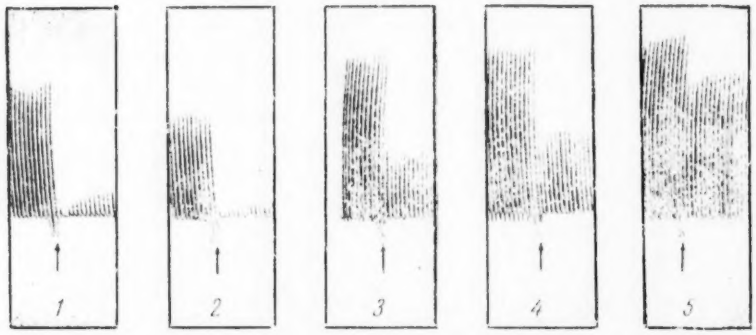
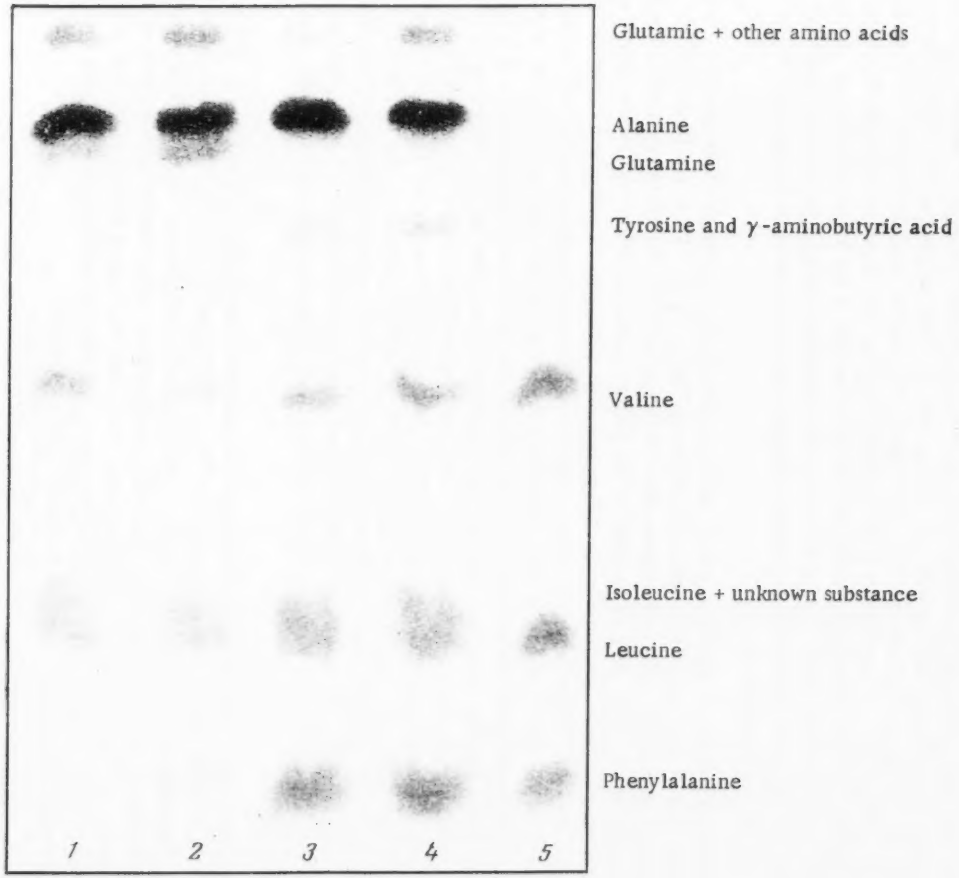


Fig. 4. Change in sensitivity of isolated frog heart ventricle to acetylcholine after the action of mercuric chloride at different temperatures: 1) action of acetylcholine at concentration $1 \cdot 10^{-6}$ g/ml in normal case; 2 and 4) sensitivity of the heart to acetylcholine after treatment of the ventricle with mercuric chloride ($4 \cdot 10^{-6}$ g/ml) for 3 minutes at 1°; 3 and 5) the same at 18°. Arrow – addition of acetylcholine in concentrations of $1 \cdot 10^{-6}$ g/ml.

These experiments show that at lower temperature the reactivity of some tissue thiol groups is lessened and the fact that in the cold the acetylcholine wave does not appear allows us to assume that the earlier rise in the curve of the cooled homogenate depends on the decreased activity of the SH groups in the choline receptors. This assumption is favored by the following data: the absence of a cold wave in titration of homogenates prepared



Typical chromatogram of extract from wheat ears. Experiment 2. Less mature ears; mixture of o-cresol + phosphate buffer, pH 6.2. 1) Introduction of H_2O into ears; 2) 0.05-M NH_4Cl; 3) 0.05-M Na-PP; 4) 0.05-M NH_4-PP; 5) spots (from top to bottom): tyrosine, valine, leucine, and PA.

from ventricles whose sensitivity to acetylcholine was fully suppressed by first giving the cardiac muscle the heat treatment at 40° (Fig. 3); the lowered reactivity of SH groups of choline receptors in whole ventricles in the cold.

Ventricles contracting from induction shocks at the rate of 30 contractions per minute were placed in a chamber at 1° and treated for three minutes with a solution of mercuric chloride at a concentration of $4 \cdot 10^{-6}$ g/ml. Then the mercuric-chloride solution was washed out, the heart was warmed to room temperature, and the change in its sensitivity to acetylcholine was determined. The cardiac muscle was repeatedly treated with mercuric chloride at the same concentration for three minutes at room temperature and from the change in sensitivity of the myocardium to acetylcholine we estimated the effectiveness of the action of mercuric chloride at 1° and at room temperature. It was shown that in the case of the action of $HgCl_2$ in the cold the sensitivity of the ventricle to acetylcholine remains almost unchanged, while at room temperature mercuric chloride has a specific antiacetylcholine activity (Fig. 4).

Thus, both the action of acetylcholine on the homogenate and cooling the homogenate to 1° give a decrease in reactivity of the same SH groups, those of the choline receptor. These results allow us to explain the absence of action of acetylcholine and impulses from the vagus nerve on the frog heart in the cold [5, 9]. Evidently in the cold there is a change in structure of the protein molecules of the choline receptors (which we have determined by the change in reactivity of the SH groups) such as to make impossible the reaction of acetylcholine with these proteins. This agrees with the earlier suggestion of the participation of SH groups in the reaction of acetylcholine with the choline receptors [4]. However, the absence of effect of acetylcholine on the reactivity of nonprotein thiol compounds [6] forces us to treat with caution the idea of direct reaction of acetylcholine with the SH groups of the choline receptors. The reaction of acetylcholine with protein SH groups of the choline receptors is evidently more complex than a simple ionic bond between the positively charged nitrogen of the acetylcholine and the electronegative SH groups of the choline receptor molecules [4]. It is possible that the competitive relation between acetylcholine and the thiol poison $HgCl_2$ is due to the fact that in the reaction of acetylcholine with the choline receptors the functional groups of the protein which adjoin the sulfhydryls also take part, and the reaction of acetylcholine with them changes the reactivity of the SH groups. On the other hand, in the reaction of mercury with SH groups, the reactivity of the functional groups which react with acetylcholine is changed.

Some unexpected results were obtained in the experiments with atropine. Preliminary addition to the homogenate of atropine at a concentration of $1 \cdot 10^{-5}$ to $1 \cdot 10^{-4}$ g/ml did not decrease the acetylcholine or the cold wave. Two suggestions are possible for this: 1) the action of atropine is connected with the structure of muscle fibers (destroying the specific structure of the muscle fibers makes the atropine ineffective); 2) the action of atropine is not connected with the occurrence of the first reaction of acetylcholine with the effector cells, that is, atropine does not react with the choline receptors, but destroys some deeper process in the reaction chain, which is responsible for the occurrence of the action of acetylcholine.

SUMMARY

Under the influence of acetylcholine there is decreased reactivity of part of the SH groups of frog heart-ventricle homogenate which can be titrated by the mercurimetric method; this depends on the reaction of acetylcholine with the choline receptors. Thus, the reaction of acetylcholine with the choline receptors proceeds not only in the whole heart muscle, but also in the tissue homogenates.

LITERATURE CITED

[1] Kh.S. Koshtoyants, Fiziol. Zh. SSSR 36, 92 (1950).

[2] T.M. Turpaev, Biokhimiya 23, 71 (1958).*

[3] Kh.S. Koshtoyants and T.M. Turpaev, Doklady Akad. Nauk SSSR 54, 181 (1946).

[4] T.M. Turpaev, Biokhimiya 20, 456 (1955).

[5] T.M. Turpaev, Doklady Akad. Nauk SSSR 102, 323 (1955).

[6] S.N. Nistratova and T.M. Turpaev, Proc. Conf. Thiol Compounds in Medicine [in Russian] (Izd. AN UkSSR, Kiev, 1958) [in press].

*Original Russian pagination. See C.B. translation.

[7] M. Kolthoff, W. Strics and L.L. Morren, Analyt. Chem. 26, 366 (1954).

[8] S.N. Nistratova, Proc. Conf. Thiol Compounds in Medicine [in Russian] (Izd. AN UkSSR, Kiev, 1958) [In press].

[9] V.B. Boldyrev, Tr. Leningr. O-va Estestvoispyt. 76, 480 (1937).

[10] M. Muriyama, J. Biol. Chem. 228, 231 (1957).

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